Adaptation of thermal scavenging ants to severe heat- conditions

Thesis · December 2018

DOI: 10.13140/RG.2.2.10091.67363

1 author:

Quentin Willot

16 PUBLICATIONS **187** CITATIONS

SEE PROFILE

FACULTÉ
DES SCIENCES

UNIVERSITÉ LIBRE DE BRUXELLES

Adaptation of thermal scavenging ants to severe heat-conditions

Adaptation des fourmis désertiques aux conditions de températures extrêmes

Thesis presented by Quentin Willot
with a view to obtaining the PhD Degree in Sciences (Docteur en Sciences)
Academic year 2018-2019

Supervisor: Professor Serge Aron

Co-supervisor: Professor Cyril Gueydan

Evolutionary Biology & Ecology

Thesis jury :

Bruno André (Université libre de Bruxelles, Chair)
Denis Fournier (Université libre de Bruxelles, Secretary)
Patrick Kestemont (Université de Namur)
Xim Cerdà (Spanish National Research Council)

fnrs
LA LIBERTÉ DE CHERCHER

Abstract

Thermal scavenging is a unique behavior restricted to a few desert ant genera. Workers are among the most thermotolerant land animals known to this day, being able to survive body temperatures of sometimes more than 50°C for several minutes. Making use of their remarkable heat-hardiness, they search for food in plain day, a feat that other desert creatures cannot accomplish. They mostly feed on the corpses of heat-stricken, less tolerant arthropods that were unable to survive the blazing sun of the midday desert. Thermal scavenging has evolved independently at least three times in distantly related genera, geographical well segregated inside the different deserts of the world. First, the *Cataglyphis* genus ranges from the Sahara Desert and extends its distribution to reach minor Asia through the Mediterranean Basin. Second the *Ocymyrmex* genus can be found in the Namib and Karoo deserts of southern Africa, extending its range to eastern Africa savanna plains. Finally, the *Melophorus* genus can be found in Australia, with thermal scavenging species distributed in the central desert of the outback region.

While this impressive behavior was already well-described by the start of this PhD project, little was known about the mechanisms supporting the remarkable heat-tolerance of workers. Using biophysical and physiological approaches in *Cataglyphis* and *Ocymyrmex*, we've been able to pinpoint key aspects underlying stress tolerance in those genera. First, from a biophysical standpoint, the Sahara silver ant *Cataglyphis bombycina* is covered with a unique and dense array of prismatic hairs reflecting visible wavelengths by total internal reflection. This allows reflection of up to 50% of the incident sunlight energy, thus shifting down the ant's thermal equilibrium and sparing its body a few critical degrees. Second, in a comparative framework, we found numerous genes involved with critical cellular processes to be constitutively expressed or strongly up-regulated to heat in thermal scavenging ants, while their orthologs were not in mesophilic species. Those processes, such as molecular chaperoning, cell-cycle regulation, energy metabolism and muscular functions are keys that allow those ants to meet the higher requirement needed to scavenge for food at both stunning speed and under extreme heat-pressure.

Overall, this work investigates the physiological and biophysical basis enabling thermal scavenging ants to survive extreme heat conditions. It provides a deeper understanding of cellular heat-tolerance pathways in non-model animals and contribute to our knowledge of life's adaptation to extreme conditions.

Acknowledgments

I would like to express my deepest gratitude to those who helped me bear through this project. First, to my supervisors Serge Aron and Cyril Gueydan for their exceptional investment and support. Second, to the members of the jury, Bruno André, Xim Cerdá, Denis Fournier and Patrick Kestemont that kindly accepted to review my work. My sincere thanks also go to colleagues, family and friends who all added their share on the scientific quality and human experience of those last four years: Sarah Chérasse, Stéphane Cherrier, Hugo Darras, Franscisco Davila, Jean-Christophe de Biseau d'Hauteville, David Bolsee, Nadège Delacourt, Rosie Dawaliby, Jean-Francois Flot, Erik T. Frank, Cédric Govaerts, Laurent Grumiau, Pierre Antoine Guérry, Peter hawkes, Simon Hellemans, Chedly kastally, Alexandre Kuhn, Esra Kaymak, Christopher Koosemans, Laetitia Marenne, Abdelkarim Nazih, Morgan Pearcy, Christian Peeters, Remy Perez, Johanna Elizabeth Romero Arias, Priscilla Simonis, Romuald Soin and Guy Vandenbussche and finaly my parents, Nicole Periquet and Yves Willot.

This work was supported by grants from the Université Libre de Bruxelles and the FRS-FNRS (Fonds national pour la recherche scientifique).

Table of content

Introduction

A. thermal scavenging ants as a model for understanding heat-tolerance

Thermal scavenging in ants

In essence, most regions labeled as deserts are hostile to life regardless of their temperatures due to their lack of available free water. However, hot deserts create especially difficult conditions, combining excessive heat with lack of moistures[1]. One of the most common means for desert animals to avoid such stresses is to have crepuscular or nocturnal activities[2]. By retreating during the day, often underground, they can evade the midday sun and the worst of the conditions that deserts have to offer.

This is not the case for all desert inhabitants though. Thermal scavenging ants have developed the exact opposite behavior: foraging for food in plain day only, exploiting windows of temperatures unbearable for other creatures. This behavior has emerged in several genera of ants distributed across arid regions of the world, to reach its most spectacular forms in desert species[3-6]. This requires from foragers to be able to survive the midday desert heat, which is no small feat. Workers need to tolerate huge loads of heat-stresses when exiting their cooler nests to run with intense metabolic activities on the burning ground, while seeking the corpses of other, less tolerant arthropods that died of heat stroke. In the Sahara Desert, air temperatures can reach as high as 50°C in summer midday hours, and ground temperatures can reach an impressive 70°C. Nevertheless, this period is actively exploited by the inhabiting thermal scavenging seeking for their sustenance.

So far, thermal scavenging behavior has been documented for: (I) the *Cataglyphis* genus from North Africa, Mediterranean region and minor Asia[6], (II) the *Ocymyrmex* genus in the Namib[7], and (III) the *Melophorus* genus in the Australian outback[8] (Fig.1A). While different species inside a genus differ in terms of distribution and environmental temperature exposures, they share similar morphological traits such as long legs to elevate their body away from the ground, high-running speed, and slender appearances (Fig.1B, D, F). Unsurprisingly, thermal scavenging ants are also among the most thermotolerant land animals known to this day, being able to survive body temperatures of sometimes more than 50°C for several minutes[8-11]. It is to be remembered though that a rich and diversified ant fauna is to be found in hot-desert and arid regions, but most species have nocturnal peaks of activity receding during the warmest part of the day. Thermal scavenging ants on the contrary, exploit this midday window, only to hide either when temperature drop down or become unbearable, even for them[12].

Because coping with stressful temperature conditions is so critical to thermal scavenging ant ecology, they have developed elaborated means of survival. Examples of some of those strategies for the Sahara silver ant *Cataglyphis bombycina* are given in Fig.2. Here, two main classes of adaptations can be separated: those aimed at shifting down the thermal equilibrium of the insect by leveling down its potential energy intake from its surroundings (avoiding temperatures), and those aimed at tolerating elevated body temperatures from a physiological and cellular perspective. The two are complementary for thermal scavenging ants to maximize survival to scorching heat.

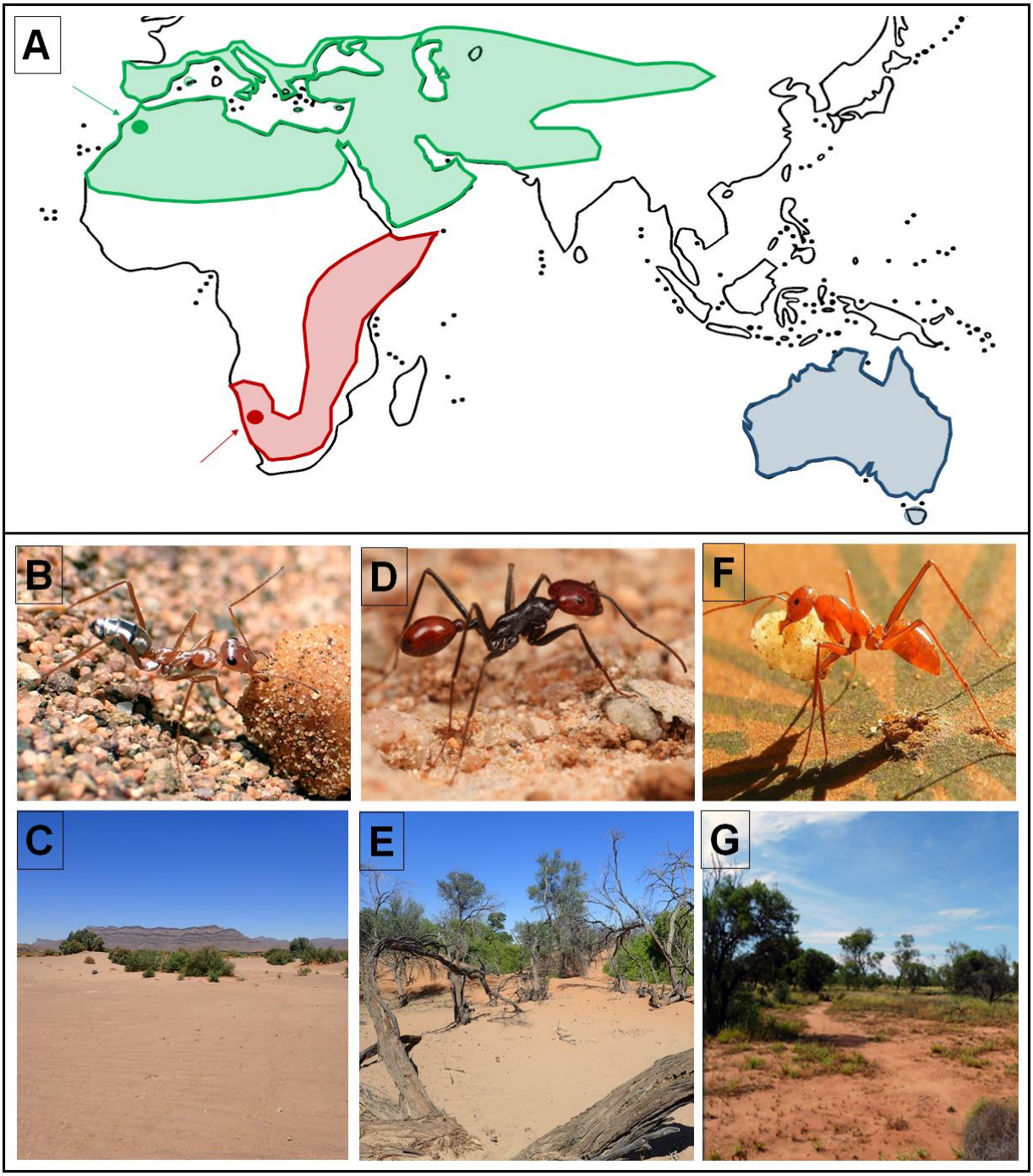

Figure 1. Distribution, morphology and habitat of thermal scavenging ant genera. A. Distribution of the *Cataglyphis* genus (green)[13], *Ocymyrmex* genus (Red)[14], and *Melophorus* genus (blue)[15], plus localities of collection of the main species studied in this thesis: *Cataglyphis bombycina*, solid green dot (Chapter I, II, and III) and *Ocymyrmex robustior*, solid red dot (Chapter IV). **B.** *C. bombycina* worker and **C.** its habitat in the sand dunes of the Dràa Valley, southern Morocco. **D.** *O. robustior* worker and **E.** its habitat in the Kuiseb river bed, Gobabeb, Namibia. **F.** *M. bagoti* worker and **G.** its semi-arid habitat in Simpson's Gap, Australia. © Willot. Q (BCE), Ducan. R (D), Wystrach. A (F), Legge. E (G).

Shifting down thermal equilibriums: understanding heat-budgets

The internal temperature of arthropods in sunlight is determined by the balance between radiative and convective heat exchanges with the environment. This is a dynamic equilibrium that can be shifted, for which the heat associated with metabolism and evaporation is normally insignificant[16]. The radiation load is received both from the sun and by reflexion from the ground. In particular, heating due to solar radiation is affected by the color of the animal (50% of the cumulative sunlight energy is made of visible wavelengths), and there may be variations up to 50% in the total radiation load due to this factor alone. Convective heat loss depends upon the shape of the animal, its relative wind-speed and the animal's orientation to it[16]. Thus, in order to understand what amount of body temperature thermal scavenger ants are exposed to, one has first to determinate the relative contribution of all those factors to their heat-budgets.

First, dissipation of excessive body heat through radiative exchanges (that is, moving to an area where air temperature is lower than body temperature allowing heat-dissipation) is an actively used process by thermal scavenging ants. Foragers exploit thermal refuges when they can, such as patches of shade or high grasses, before continuing intermittent foraging activities (Fig.2)[11]. Thermal scavenging ants are also morphologically well adapted to their activity, with long legs elevating their vital functions away from the burning sand (a 5-10°C drop of temperatures can be observed 4mm above ground, which is ant-height[11]) and allowing extremely fast running speed as compared to their size (of up to 1m/s for *C. bombycina*). This speed serves two purposes. First, it enables the ant to minimize the time spent outside the nest foraging and bringing back food. Second, it allows workers to cool themselves via a forced convective heat-loss during locomotion (in other words, their running speed creates a relative airflow that cools the ant's body)[17]. While those adaptations are common to all thermal scavenging ants, a unique feature of *C. bombycina* is its silver appearance. This color stems from the dense array of triangular-shaped hairs covering the dorsal face of workers (Fig.1B; Fig.2). Because of this triangular cross-section, hairs act much like prisms: lights enter one face of the hair and is reflected almost totally through total internal reflection (TIR) on the basal face of the triangle[18,19] (Chapter I). This strong and directional reflection of visible wavelengths accounts both for the ant silver sheen and for thermoregulatory properties. By minimizing energy absorption in the visible and near infra-red ranges of the spectrum, workers can effectively spare a few critical degrees. Altogether, those behavioral and morphological adaptations give thermal scavenging ants enhanced control on their radiative and convective heat-exchanges, shifting down their thermic equilibriums and keeping their body temperature supportable during their outside activities.

So, what are the real body temperatures thermal scavenging ants experience during their foraging trips? *In vivo* body temperature of foragers is inherently elusive to measurement because it is heavily impacted by the convective cooling effect of the ants' natural running speed. Thus, relying on *in vitro* experiments mimicking an average *Ocymyrmex robustior* foraging trip, estimations pointed out that a worker body temperature could reach approximately 49°C[17]. This is close to the species' critical thermal maximum (CT_{max}) of 51.2°C[7] (CT_{max} can be defined here as the temperature onset of loss of muscle coordination and heat-induced coma). Overall, it is indeed argued that thermal scavenging ants should forage at body temperatures close to their own thermal limits[10-12]. CT_{max} of species should therefore be good indicators of the upper range of internal temperature those insects likely experience in natural foraging conditions.

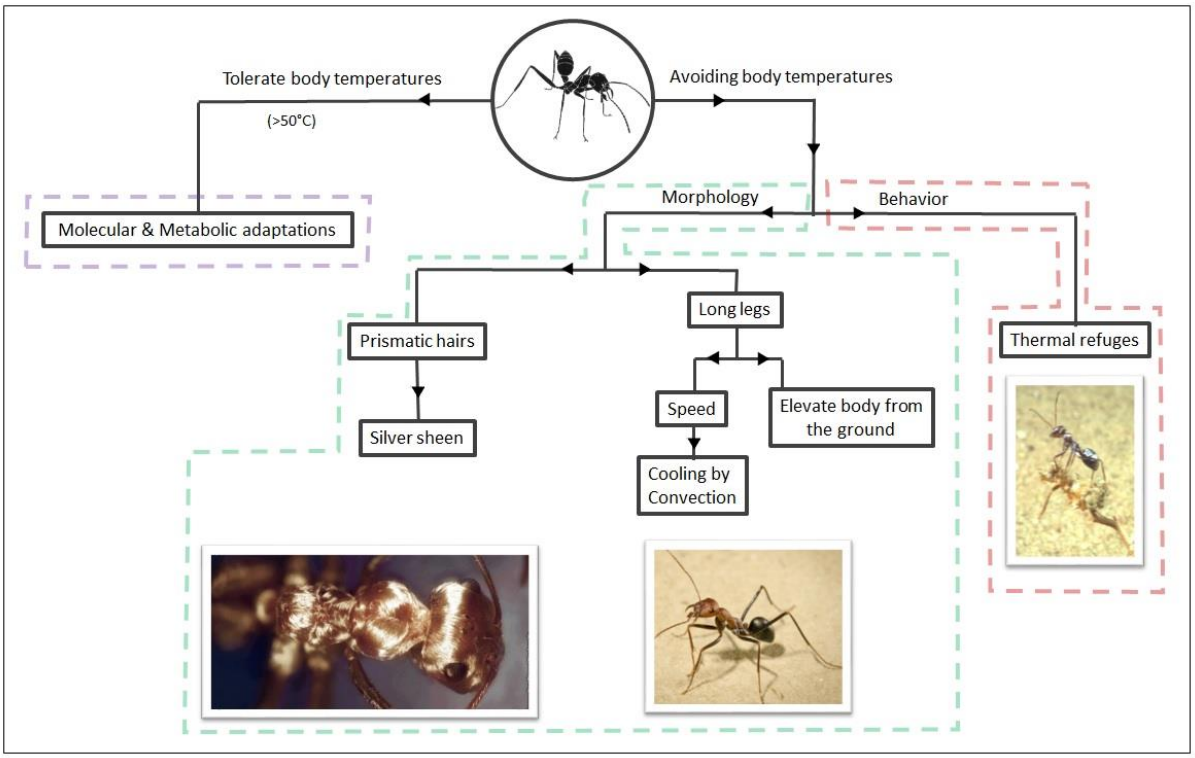

Figure 2. Schematized overview of the main known strategies of *C. bombycina* to survive heat-stress. Workers use behavioral clues (thermal refuges)[11] as well as morphological adaptations (elevating their vital functions away from the burning ground[11], high-running speed,[11,17] and a reflective coat of hairs to reduce their thermal equilibrium while foraging[18,19]). These are complemented by active molecular and metabolic adaptations at the cellular level[20-22], allowing *C. bombycina* to still experience and survive daily temperatures that would be lethal to most other animals. © Mangan. M, Shi. N.

Heat-tolerance adaptations in thermal scavenging ants

The lethal body temperature for most mammals, including humans, is 42°C. In insects, the average CT_{max} of 173 species has been estimated around 43°C[23]. Thermal scavenging ants, on the other hand, dwarf those numbers from the very top of the podium with CT_{max} of 50°C and more (*Ocymyrmex robustior*, 51.2°C; *Cataglyphis bombycina*, 53.6°C; the record so far being *Melophorus bagoti* with a CT_{max} of 56.6°C)[7,8,11]. However, it is to be noted that measurements of CT_{max} are actually poorly reproducible and quite dependent on the methodological context and thermal history of individuals (*i.e* if individuals have acclimated to warmer temperatures or not prior to experimentation, leading to up-regulation of heat-related genes and increased tolerance). CT_{max} thus need to be considered only as an estimation of upper thermal limits[23,24]. Nevertheless, if thermal scavenging ants indeed experience and survive body temperatures close to their CT_{max} for several minutes while foraging, they can then be considered so far as among the most heat-tolerant terrestrial animals known.

In order to survive those conditions, thermal scavenging ants need to have developed specific physiological and molecular adaptations to tolerate transient burst of intense heat-stresses. However, only a single work prior to the beginning of this thesis, dating from 1995, has pushed forward our knowledge of the physiological basis supporting heat-tolerance in thermal scavenging ants. Using western blot, Gehring & Wehner[20] showed that workers of *C. bombycina* accumulate Hsp70 constitutively and without any heat-exposure, suggesting that it would spare workers the need to adapt to sudden exposure to elevated temperature. The lack of investigation in this area might partially be due to the fact that thermal scavenging ants (and

ants in general) are rather inadequate models for molecular biology investigations. They have complex habits with year-long breeding cycles and difficult long-term requirements to meet in captivity. This makes impossible the development of genetically distinct strains that maintain over generations. Most of the molecular and cellular biology data surrounding arthropods thus stem from *Drosophila* species which, as opposed to ants, make good models for such topics of investigation. Hopefully, since 1995, advances in techniques have made exploration cellular bilogy readily accessible in previously non-model organism, including ants[25]. For example, in the xerothermic species *Formica cinerea* found in open temperate habitats across northern Europe, quantitative real-time PCR recently allowed to correlate *hsp* genes expression and temperature of worker exposure in lab experiments[26]. The same line of investigation has been reported in the harvester ant *Pogonomyrmex barbatus*[27], and the woodland species *Aphaenogaster picea* and *Aphaenogaster rudis*[27,28]. However, it has to be considered that those ants do not display a typical thermal scavenging behavior, and that their heat-tolerance is much lower than *Cataglyphis*, *Ocymyrmex* and *Melophorus* genera. Thus, while the last decades have been fertile grounds for exploring the behavioral and morphological aspects of thermal scavenging, this thesis, alongside with a few other works, picks up after the first work of Gehring and Wehner and dives deeper into its molecular and physiological considerations. It should open the path for further work, and it is to be hoped that this field of investigation will help to push forward our understanding of the physiological and molecular adaptations developed by life to thrive in harsh conditions.

References

1. Schmidt-Nielsen, K. (1964). Desert animals. Physiological problems of heat and water. *Desert animals. Physiological problems of heat and water.*
2. Brown, G. W. (Ed.). (2013). *Desert biology: special topics on the physical and biological aspects of arid regions*. Elsevier.
3. Wehner, R. (1987). Spatial organization of foraging behaviour in individually searching desert ants, Cataglyphis (Sahara desert) and Ocymyrmex (Namib desert). In *From individual to collective behaviour in social insects: les Treilles Workshop/edited by Jacques M. Pasteels, Jean-Louis Deneubourg*. Basel: Birkhauser, 1987.
4. Muser, B., Sommer, S., Wolf, H., & Wehner, R. (2005). Foraging ecology of the thermophilic Australian desert ant, Melophorus bagoti. *Australian Journal of Zoology*, *53*(5), 301-311Wehner, R., &
5. Wehner, S. (2011). Parallel evolution of thermophilia: daily and seasonal foraging patterns of heat-adapted desert ants: Cataglyphis and Ocymyrmex species. *Physiological entomology*, *36*(3), 271-281.
6. Boulay, R., Aron, S., Cerdá, X., Doums, C., Graham, P., Hefetz, A., & Monnin, T. (2017). Social life in arid environments: the case study of Cataglyphis ants. *Annual review of entomology*, *62*, 305-321
7. Marsh, A. C. (1985). Thermal responses and temperature tolerance in a diurnal desert ant, Ocymyrmex barbiger. *Physiological zoology*, *58*(6), 629-636.
8. Christian, K. A., & Morton, S. R. (1992). Extreme thermophilia in a central Australian ant, Melophorus bagoti. *Physiological Zoology*, *65*(5), 885-905.
9. Marsh, A. C. (1985). Microclimatic factors influencing foraging patterns and success of the thermophilic desert ant, Ocymyrmex barbiger. *Insectes Sociaux*, *32*(3), 286-296.
10. Cerdá, X., Retana, J., & Cros, S. (1998). Critical thermal limits in Mediterranean ant species: trade-off between mortality risk and foraging performance. *Functional Ecology*, *12*(1), 45-55.
11. Wehner, R., Marsh, A. C., & Wehner, S. (1992). Desert ants on a thermal tightrope. *Nature*, *357*(6379), 586.
12. Cros, S., Cerdá, X., & Retana, J. (1997). Spatial and temporal variations in the activity patterns of Mediterranean ant communities. *Ecoscience*, *4*(3), 269-278.
13. Radchenko, A. G. (2001). The phylogeny and faunogenesis of the genus Cataglyphis Foerster (Hymenoptera, Formicidae). *Entomological Review*, *81*(8), 951-958.
14. Bolton, B., & Marsh, A. C. (1989). The Afrotropical thermophilic ant genus Ocymyrmex (Hymenoptera: Formicidae). *Journal of Natural History*, *23*(6), 1267-1308.
15. Heterick, B. E., Castalanelli, M., & Shattuck, S. O. (2017). Revision of the ant genus Melophorus (Hymenoptera, Formicidae). *ZooKeys*, (700), 1.
16. Parry, D. A. (1951). Factors determining the temperature of terrestrial arthropods in sunlight. *Journal of Experimental Biology*, *28*(4), 445-462.
17. Marsh, A. C. (1985). *Aspects of the ecology of Namib Desert ants* (Doctoral dissertation, University of Cape Town).
18. Shi, N. N., Tsai, C. C., Camino, F., Bernard, G. D., Yu, N., & Wehner, R. (2015). Keeping cool: enhanced optical reflection and heat dissipation in silver ants. *Science*, aab3564.
19. Willot, Q., Simonis, P., Vigneron, J. P., & Aron, S. (2016). Total internal reflection accounts for the bright color of the Saharan silver ant. *PloS one*, *11*(4), e0152325.
20. Gehring, W. J., & Wehner, R. (1995). Heat shock protein synthesis and thermotolerance in Cataglyphis, an ant from the Sahara Desert. *Proceedings of the National Academy of Sciences*, *92*(7), 2994-2998.
21. Willot, Q., Gueydan, C., & Aron, S. (2017). Proteome stability, heat hardening, and heat-shock protein expression profiles in Cataglyphis desert ants. *Journal of Experimental Biology*, jeb-154161.
22. Willot, Q., Mardulyn, P., Defrance, M., Gueydan, C., & Aron, S. (2018). Molecular chaperoning helps safeguarding mitochondrial integrity and motor functions in the Sahara silver ant Cataglyphis bombycina. *Scientific reports*, *8*(1), 9220.
23. Hoffmann, A. A., Chown, S. L., & Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they?. *Functional Ecology*, *27*(4), 934-949.
24. Terblanche, J. S., Deere, J. A., Clusella-Trullas, S., Janion, C., & Chown, S. L. (2007). Critical thermal limits depend on methodological context. *Proceedings of the Royal Society of London B: Biological Sciences*, *274*(1628), 2935-2943.
25. Trible, W., Olivos-Cisneros, L., McKenzie, S. K., Saragosti, J., Chang, N. C., Matthews, B. J., ... & Kronauer, D. J. (2017). Orco mutagenesis causes loss of antennal lobe glomeruli and impaired social behavior in ants. *Cell*, *170*(4), 727-735.
26. Ślipiński, P., Pomorski, J. J., & Kowalewska, K. (2015). Heat shock proteins expression during thermal risk exposure in the temperate xerothermic ant Formica cinerea. *Sociobiology*, *62*(3), 457-459.

27. Nguyen, A. D., Gotelli, N. J., & Cahan, S. H. (2016). The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC evolutionary biology*, *16*(1), 15.
28. Cahan, S. H., Nguyen, A. D., Stanton-Geddes, J., Penick, C. A., Hernáiz-Hernández, Y., DeMarco, B. B., & Gotelli, N. J. (2017). Modulation of the heat shock response is associated with acclimation to novel temperatures but not adaptation to climatic variation in the ants Aphaenogaster picea and A. rudis. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *204*, 113-120.

B. Cellular adaptations to cope with heat-stress

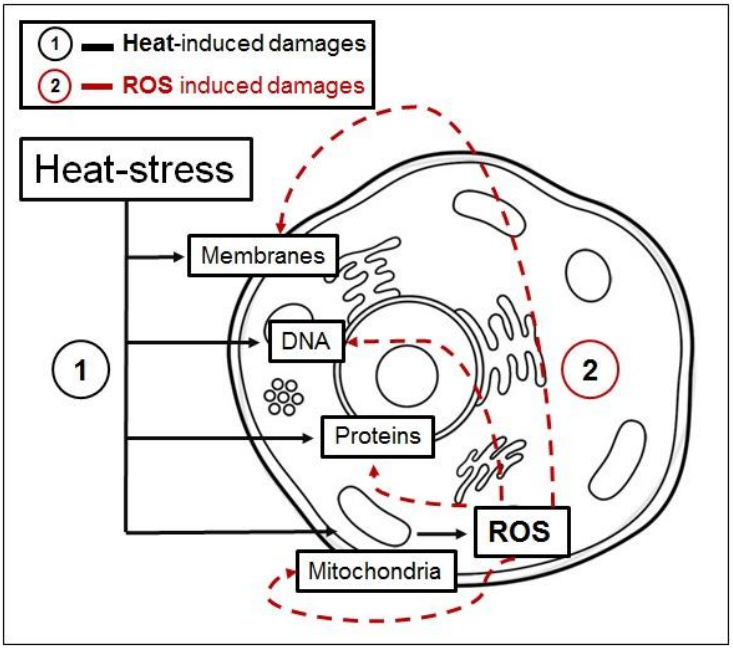

Figure 3. Scheme of the main macromolecular target for damages through heat and oxidative stresses in eukaryotic cells. Mitochondria are the primary source of reactive oxygen species (ROS) overproduction due to heat-disruption of aerobic metabolism.

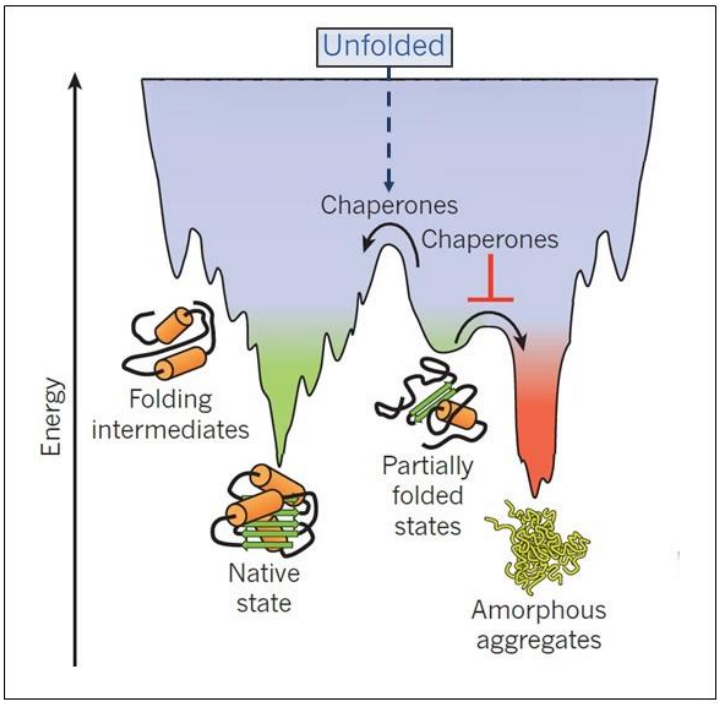

Figure 4. Two-dimensional, simplified overview of the free-energy surface that unfolded proteins explore as they move towards their native state. Proteins can acquire kinetically trapped conformations that need to traverse free-energy barriers to reach a favorable path. *In vivo*, these steps are assisted by molecular chaperones (see dedicated sub-chapter: *Heat-shock proteins: guardians of macromolecular integrity*). Non-favorable conformations can include the formation of amorphous aggregates. Modified from Hartl *et al., 2011*[19].

Thermal variation and cellular homeostasis

One of the many distinctions that set life apart from other physical entities is its ability to sustain biological processes segregated from their surroundings[1].Although cells can insulate themselves from external compounds that could disrupt their inner balance, they have limited capacities in doing so from variation of temperatures[2]. In turn, molecular agitation (*i.e* heat) has critical impacts on chemical kinetics and thermodynamic equilibriums, and thus on the ability of life to sustain itself at peak efficiency[3].

This is especially true when it comes to the stability of cellular macromolecules. Macromolecules are commonly referred to as very large molecules (polymers) created by the polymerization of smaller molecules (monomers)[4]. Important examples of macromolecules are proteins, formed by assemblages of amino acids, DNA and RNA which are created by assemblages of nucleotides, and cellular membranes built by phospholipid bilayers. They all possess three-dimensional structures that define their biological functions. However, these structures have evolved to be stable within narrow ranges of temperatures and can be easily disrupted by shifts in molecular agitation, with subsequent loss of biological activity[5]. Because of this, a non-optimal temperature can lead to systemic failures of cellular metabolism followed by cell death. In pluricellular organisms, cellular loss can lead to tissue damages, organ failures and, in major cases, a deadly collapse of biological functions. Maintaining macromolecular stability despite changing thermal conditions has thus been one of the very first challenge life had to overcome in order to thrive on Earth[6].

To understand how and to what extent molecular agitation can impact macromolecular stability, one must first understand that macromolecular three-dimensional structures result from thermodynamic equilibriums. As with all equilibriums, this one can be moved. The main factor balancing macromolecular stability is intermolecular interactions[7]. This force mostly englobes in the present context hydrogen bonding, dipole-dipole interactions, and van der Waals forces. For example, amino acids can create hydrogen bonding between each other and with molecules of their surroundings, depending on the polarity of their lateral chains' structure. This is important to understand how a seamless change in a protein amino-acid sequence can lead to shifts of its 3-D conformation. Because those new amino acids will create different intermolecular interactions with the rest of the protein's amino acids, as well as with the surrounding medium, the resulting equilibrium dictating the peptide conformational stability will change. Intermolecular interactions rely on electrostatic forces that are themselves largely dependent on molecular agitation. Thus, the impact of molecular agitation on macromolecular stability can be seen as a chain-effect of events: variations of molecular agitation lead to variations in the electromagnetic strengths dictating intermolecular interactions, which in turn leads to variations in the thermodynamic equilibrium dictating the macromolecule's spatial conformation. Here, a special case should be made concerning the amino-acid cysteine that can create covalent disulfide bonds with each other. Covalent bonds require more energy to break than intermolecular forces, allowing disulfide bonds to locally enhance protein stability to heat-stress in a way intermolecular forces couldn't achieve. However, on a global scale, disruption of intermolecular forces by heat-stress does impact all macromolecules, whose level of structural sensitivity to heat varies depending on their own chemical properties and surroundings (Fig.3). Heat-stress causes proteins to lose their structures and thus to lose their biological activities, which is known as the denaturation process (Box 1)[9]. Unfolded proteins then need active help to return to their native state (*i.e* their intended spatial conformation; Fig.4). DNA and RNA secondary structures also denaturate to heat-stress (Box 2)[10], as well as cell membranes whose fluidity depends on temperature (Box 3)[11-14]. Those three types of macromolecules are basic bricks to life as we know it, and their relative contribution to cellular integrity in a context of heat-stress will be approached repeatedly throughout this work.

Box 1: The implications of molecular agitation on protein stability

In proteins, the main forces behind intra and intermolecular interactions are by order of thermodynamic strength: disulfide covalent bonds formed between two cysteine residues (intramolecular forces), hydrogen bonds involving OH and NH groups of amino acids, and dipole attractions (intermolecular forces). Intermolecular interactions also occur between the outer-layer of a peptide's amino acids and the surrounding solvent (H_2O in case of intracellular medium). Because water is a strong polar medium, it will exert direct repulsion over hydrophobic amino-acids, thus pushing them away from the surface of the protein while at the same time, favoring interactions with hydrophilic residues. From this balance between attractions and repulsions stems the protein conformational structure. By disrupting this thermodynamic balance, shifts in molecular agitation will cause proteins to lose their structure and to denaturate. Protein denaturation causes exposure of their inner hydrophobic residues to the water medium, which reduce their solubility. This traps proteins into non-native conformational states that need active chaperones assistance to correct and causes the apparition of cytotoxic aggregates that are difficult to resolve and that precipitate (Fig.4). General protein misfolding in cells will lead to disruption of the cytoskeleton structure, loss of metabolism and signaling pathways, loss of DNA and RNA structures, organelles disorganization, and finally, apparition of stress granules made of aggregated proteins and large RNA-protein structures, all of which have severe and potentially lethal effects[16].

Box 2: The implications of molecular agitation on nucleic acids secondary structures

First, in DNA/RNA, the main forces behind intra and intermolecular interactions are hydrogen bonds involving OH and NH groups of nucleotides, and dipole attractions. Because the electrostatic forces behind those interactions are dependent on molecular agitation, temperature will exert a direct impact on DNA/RNA secondary structure conformational stability. Second, it must be considered that most of the cell control over DNA and RNA topology is also mediated through interactions with proteins. For example, ribosomes are complex of several RNA molecules embedded with proteins, while DNA is bound in the nucleus to histone proteins to form nucleosomes. Interactions between DNA/RNA and proteins also rely on intermolecular forces and can thus be disrupted by molecular agitation as well. Practically, RNA and DNA secondary structures are involved in protein synthesis, gene transcription, replication, and DNA maintenance. Protein synthesis, cellular division and gene expression are consequently highly thermosensitive aspects of cellular biology.

Box 3: The implications of molecular agitation in cell membranes fluidity

Biological membranes are composed of phospholipid bilayers embedded with various amounts of sterol molecules and proteins. Membranes are not static: phospholipids constantly move around, arranging and rearranging while the embedded proteins, alongside with other biomolecules, can diffuse and travel along the membrane. The extent of molecular motion within a lipid bilayer is referred to as the fluidity of the membrane and is directly impacted by temperatures (Fig.5). An optimal dynamism, that can be loss due to excessive temperature, is critical for membrane functions such as signal detection, selective transport, and maintenance of the transmembrane potential. In addition, reactive oxygen species that are over-produced during heat-stress damage membranes through lipid peroxidation and protein oxidation. Extensive lipid peroxidation causes in turn additional issues for membrane fluidity. Over fluidization and damages to membranes severely impairs cellular homeostasis and can lead to lethal ruptures and releases of cell contents.

On top of macromolecular damages through disruption of electromagnetic forces, another major issue stemming from heat-stress is the over production of toxic intracellular reactive oxygen species[15] (ROS; Fig.3). ROS are highly reactive chemical species containing oxygen that, in a biological context, are formed mainly in the mitochondria as a natural byproduct of aerobic oxygen metabolism[17]. However, during times of environmental stresses (such as exposition to ultraviolet radiation, to toxics, or to heat-stress), ROS levels can increase dramatically, leading to oxidative damages[18]. This holds several consequences: (1) the ROS-mediated oxidation of macromolecules both inside and outside of the mitochondria (lipids, DNA and proteins) add a second and potentially lethal layer of damages to the one caused by heat-shock (Fig.3), (2) aerobic metabolism is impaired and ATP production plummets, which reduce the ability of cells to sustain their metabolism and (3) mitochondria can be damaged beyond repair, which leads to their recycling, reduction in number, reduced energy production and overall difficulties for cells to efficiently recover from heat-shock[15-18].

In conclusion, shifts in thermal regime perturb chemical kinetics and the three-dimensional structure of macromolecules, as well as causing indirect damages to cell structures through the over-production of aggressive ROS. Those adverse effects opposed by thermal stress to cellular homeostasis are challenging; most essential aspects of cellular biology are impaired, which can lead to a chain-failure of cellular processes and death. Hopefully, mechanisms stabilizing macromolecular structures despite thermal variations and oxidative stress have been selected, enabling organisms to adapt to the ranging conditions they've encountered over the course of their evolutive history.

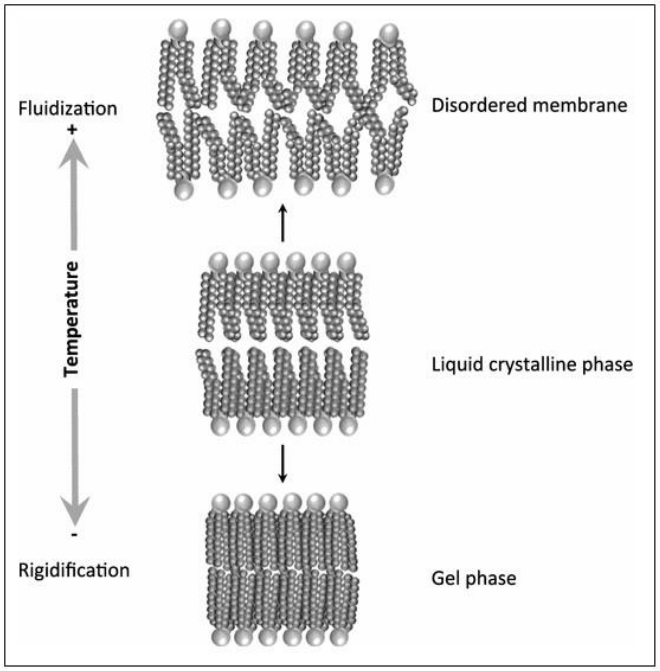

Figure 5. Schematic representation of variations in membrane structure exposed to above or suboptimal temperatures. Low temperatures cause "rigidification" of membranes, whereas high temperatures cause "fluidization" of membranes which increases their permeability[20]. From Los & Murata, 2004.

Resistance and tolerance adaptations to cope with shifts in temperatures

Environmental conditions, and especially thermal environments, are inherently fluctuating in most biomes. As a response, a wide array of adaptations to increase macromolecular stability and cope efficiently with shifts in thermal regime appeared early in evolution[5]. Here, it is especially important to make a distinction between passive adaptations aimed at shifting the optimal temperature for macromolecular stability up or down (*i.e* adapting to a constant high or low temperature), and active adaptations still deployed to minimize damages when outside this optimum (*i.e* to tolerate shifts in temperatures). These two forms of adaptations relate to the distinct mechanisms of resistance and tolerance[21]. Both mechanisms are complementary. However, resistance implies that damages do not occur, and that the organism will not have to spend energy on repair and stabilizing mechanisms, being thus perfectly adapted to a given temperature. On the contrary, tolerance implies that damages are occurring and that they need to be kept in line through an active and energetically costly maintenance.

Numerous examples of resistance adaptations can be found in extremophiles bacteria and archaea[22]. In those organisms, most aspects biochemical lifestyles have been adapted to nullify the potential damages caused by high temperatures (as well as other abiotic stresses). Proteins have evolved to be functional in elevated thermal conditions, mostly by having naturally loose structures retaining both solubility and biological activity[23]. DNA structural stability is increased through increases in G-C contents and presence of heat-adapted proteins, and the biochemical nature of lipids forming membranes is modified to confer resistance to heat-induced hydrolysis (ether lipids can be found in hyperthermophiles, as opposed to ester lipids found in mesophilic organisms)[24]. In addition, most hyperthermophiles are either strict anaerobics or facultative microaerophilics (*i.e*, growing at very low oxygen concentration only), thus bypassing most issues caused by heat-induced ROS production altogether[22]. Such extended resistance adaptations confer hyperthermophiles optimal growth temperatures of above 80°C, and even of above 100°C in exceptional conditions such as around deep-sea hydrothermal sources. The Archaea *Pyrolobus fumarii*, found in thermal vents 3650 meters deep in the mid-Atlantic ridge, has for example an optimal growth temperature of 106°C[25].

Resistance adaptations, as useful as they may be for shifting thermal preferences up and down, are inherently limited for preserving macromolecular integrity when still outside organisms' thermal optimum. Even hyperthermophiles can be exposed to non-optimal temperatures, causing biochemical and macromolecular stability issues[26]. Thus, in almost all organisms known so far, active tolerance mechanisms have been selected to cope with rapid changes in thermal conditions. First, when it comes to maintaining protein structural damages to a minimum, one of the most prominent responses is the heat-shock response (HSR)[16]. Because of its ancestralism, the HSR shares core mechanisms in all known taxa[27], from bacteria to higher eukaryotes. The conserved characteristic of the HSR is its reliance on the use of molecular chaperones (which are proteins that assist the folding, stability and assembly of other macromolecular structures)[19]. The most famous examples of molecular chaperones are heat-shock proteins (Hsps), whose classification and functions will be elaborated further in this chapter. Some organisms also synthesize and accumulate low molecular weight molecules, known as osmolytes, with beneficial effects on macromolecular stability[28]. Because keeping a proper membrane fluidity is essential, a known response to heat-shock is also an active regulation of membranes phospholipid composition[29].

In addition, antioxidant defenses, whose mechanisms will also be elaborated further in this chapter, are deployed to keep ROS induced damages to a minimum[30]. Finally, in higher eukaryotes, cell survival to macromolecular damages can be enhanced through modulations of autophagic and apoptotic flux pathways[31]. All those responses aim for the cells to cope as well as possible with transient shifts into non-optimal temperatures and can therefore be regrouped into a tolerance class of adaptations.

Thus, at the cellular level, resistance and tolerance are two complementary strategies. While the first has for purpose to nullify the macromolecular damages caused by on average higher or lower temperatures, the second aims to cope efficiently with the variations of temperature organisms can still be exposed to. One must keep in mind that the pinnacle of known resistance adaptations is to be found in micro-organisms that have effectively colonized the most extreme environments on Earth[22]. In eukaryotes, the frequency and biochemical extent of resistance adaptations to heat tend to level down. As opposed, the complexity of their tolerance mechanisms rises significantly[5], with comparatively complex arrays of deployed molecular chaperones, and more angles for the cell to promote its survival through precise and transient adaptation of its metabolism. Below, we will focus deeper on general tolerance mechanisms deployed by animal cells. However, it has to be considered of all potential factors contributing to heat-stress tolerance, heat-shock proteins are for historical reasons the most studied in non-model organisms[32]. They are thus far more accessible to frame in their wider context than other existing mechanisms. For this reason, both this introduction, as well as results presented in chapter II, III and IV, will mainly emphasis on Hsps.

Heat-shock proteins: guardians of macromolecular integrity

The first recorded observation of the heat-shock response dates from 1962, when Italian geneticist Ferruccio Ritossa reported a characteristic pattern of "puffing" in the chromosomes of *Drosophila* salivary glands exposed to heat (Fig.6)[33]. This observation led to the subsequent identification of heat-shock proteins (Hsps), the “puffings” being in fact located on *hsps* genes during their transcription. Since then, Hsps have been discovered to be expressed in all cellular organisms and in response to a wide array of conditions including exposition to heavy metals, hypoxia, irradiation, oxidative and osmotic stresses[34]. The common trait shared by all those stresses is their potential proteotoxic effects, in other words, their ability to directly or indirectly cause imbalances to the three-dimensional structures of proteins. As such, Hsps can be more accurately described as part of a response triggered by any form of proteotoxic stress, rather than by heat alone.

Cells proteostasis depends on the balance between protein folding and protein degradation. At one end of the spectrum, the heat shock proteins modulate proper folding and repair of either already present or newly synthesized peptides[6]. At the other end, the proteasome and autophagic pathways, as well as other lysosome-dependent proteolytic systems, function in the degradation of dysfunctional proteins[35,36]. Because Hsps assist the correct spatial folding, transport, and translocation of those newly synthesized or misfolded peptides, they prevent protein denaturation, loss of biological functions, and toxic aggregation[5,19]. Through feedback mechanisms, Hsps also assess cellular proteostasis and relay this information to pathways regulating core processes such as cell division, migration, response to stimuli and death[19,37]. Because of this widespread implication in cellular life, Hsps have become of interest to many fields of biology, such as ecophysiology (that aims to understand how organisms respond physiologically to their environmental conditions) or medical sciences on topics as diversified as cancerology, immunology, and toxicology. This chapter will be restricted to the functional aspects of Hsps in a context of heat-tolerance in animals.

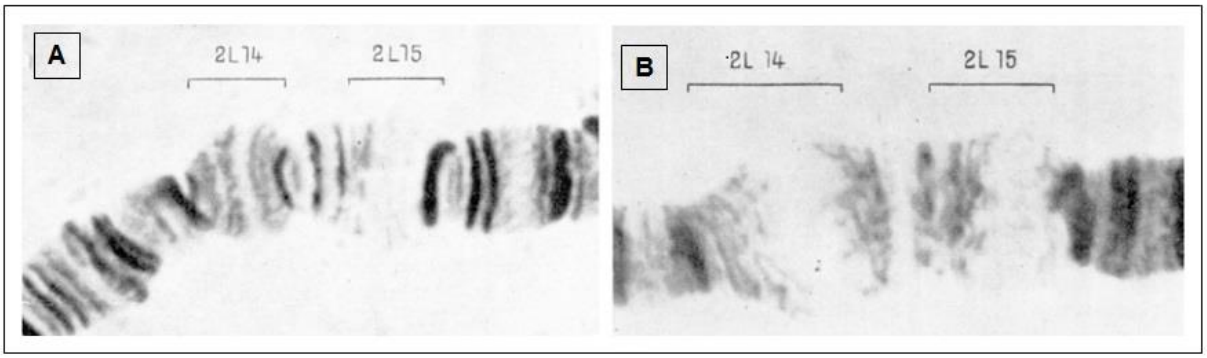

Figure 6. The 2L14 and 2L15 regions of salivary glands chromosomes of *Drosophila busckii* before and after heat-shock. A. Larvae reared at 25°C**. B.** Larvae exposed to a 1-hour heat-shock of 30°C. Chromatin decondensation is necessary for gene transcription, and a distinct "puffing" pattern appears during heat-shock at the two regions which were later identified to be loci for *hsp70* genes. Adapted from Ritossa, 1962[33].

So far, it is agreed upon that the predominant classes of Hsps in eukaryotic organisms include six broadly conserved families that are distinguished based on their respective molecular weights: Hsp100s, Hsp90s, Hsp70s, Hsp60s, Hsp40s, and Hsp20s that are small heat shock proteins (sHsps)[16,19]. While most Hsps tend to be structurally conserved across taxa, gene duplications and rearrangement in their promoter region (that dictates their potential heat-inducibility) has led to a multitude of complex, organism-specific responses with their own variant and peculiarities[38,39]. Hsps are present in the cytosol, mitochondria, endoplasmic reticulum and nucleus, although these locations vary depending on the particular protein[40]. They have functional roles that can be segregated into two non-mutually exclusive groups[16]. First, the "holdase" activity is optimized for efficient binding to misfolded proteins, mostly through enhanced interaction with hydrophobic patches. By doing so, Hsps with holdase activity prevent denatured proteins to lose their solubility and to aggregates with each other, clustering them for a more easily resolution afterward[41]. Second, the "foldase" activity, as its name indicates, is an ATP-dependent process aimed at helping the proper folding of peptides. Hsps with foldases activity often have limited capacities on their own; their abilities rely on the cooperation with other Hsps and co-chaperones, each having their distinct role in the folding machinery[42]. The relative contribution of each Hsp family to heat-tolerance mechanisms is further detailed in Box 4 to 7, and a simplified overview of their activity is given in Fig.7. Further relevant readings can be found in reviews from Richter *et al.*, 2010[16] and Harlt *et al.*, 2011[19].

In short, Hsps are essential for proper protein-folding in physiological and stressful conditions. However, because a proper proteostasis is necessary to cellular life, they have also evolved to integrate signals of protein damages into more general pathways controlling cell division, death, and response to its surroundings. The goal of the HSR and Hsps induction is to promote cell survival when exposed to proteotoxic stresses, such as heat-shock. While most Hsps tend to be structurally conserved across taxa, each organism has evolved its own peculiarities of combination when it comes to the folding machinery and its regulation. Hsps are the first cellular line of defense against heat-stress, and regardless of the considered organism, their expression levels have been repeatedly linked with thermotolerance.

Box 4: Hsp70s, Hsps40s and Hsp100s activity

The Hsp70 family is one of the most ubiquitous and conserved family with foldases activity[43]. The activity of Hsp70s is critical for protein homeostasis in stressful conditions, and regulated through interactions with several co-factors, notably Hsp40 and BAG proteins[44,45]. Because proteostasis also relies upon degradation of misfolded peptides, Hsp70s can relay damaged proteins to proteolytic systems, for example through interactions with BAG proteins[46]. Hsp70 can also integrate other modular Hsps complexes: together with Hsp40 and Hsp100, it mediates the disassembly and subsequent refolding of cytotoxic protein aggregates (Fig.7)[41,47]. In tandem with Hsp90, it regulates pathways involved in protein synthesis, cell growth, division, immune response, and death[48-51]. The Hsp70 family includes multiple structurally related proteins with cytosolic or organelle-specific localizations[52]. Interestingly, in most organisms this family has been repeatedly included among the most heat-inducible gene in response to proteotoxic stresses. This highlights the conserved, ubiquitous role of Hsp70 proteins in heat-stress tolerance and recovery.

Box 5: Hsp60s activity

Important actors of the mitochondrial folding activity are chaperonins, represented by Hsp60s in eukaryotes[19]. They are large complexes that function by globally enclosing substrate proteins up to ~60 kDa for folding[16,19]. In eukaryotes, they are restricted to chloroplasts and mitochondria[53] and are essential components of mitochondrial protection against proteotoxic stresses[54].

Box 6: Hsp90s activity

The Hsp90 family is remarkable among Hsps in a way that it has, in eukaryotes, evolved a large panel of complementary functions in cell regulation in addition to its activity in the folding machinery. More than 20 co-chaperones are known to regulate the activity of Hsp90 proteins with relation to the cell cycle and metabolism, both under stressful and normal conditions[55,56]. Hsp90s mostly seem to act downstream of Hsp70s as integrators of signaling pathways involved with protein homeostasis, cell proliferation, maturation, division, autophagy and death[57,58]. In a heat-stress survival context, Hsp90 is also a key protein in the control of the HSR: it forms a stress-sensitive complex that dissociates in case of proteotoxic stresses, releasing the transcription factor HSF1 responsible for the synthesis of other stress-induced Hsps[59]. Hsp90s thus articulate signals of macromolecular damages into other aspects of cellular life that need to be regulated to promote organisms' survival to stress (such as synthesizing other Hsps, haltering the cell-cycle or triggering autophagy and programed cell-death).

Box 7: small Hsps activity

Small Hsps members (sHsps, or Hsp20s) have notable "holdase" activities. Functionally, sHsps are ATP-independent chaperones that interact with partially folded proteins to prevent their aggregation upon stress-induced denaturation (Fig.7)[60]. They are the most widespread, and also the most poorly conserved family of molecular chaperones[61]. It seems that in addition to their ability to form complexes with denatured proteins to keep them soluble, they can also be sequestered into aggregates when denatured proteins precipitate[41]. This subsequently helps the ATP-dependent folding machinery to disaggregate and refold those proteins afterward[62]. Importantly, in animals, it has been shown that sHsps specifically help to stabilize myofibrillar proteins during stress conditions[63-65] and provide further assistance in myofilaments maintenance through selective recycling[51].

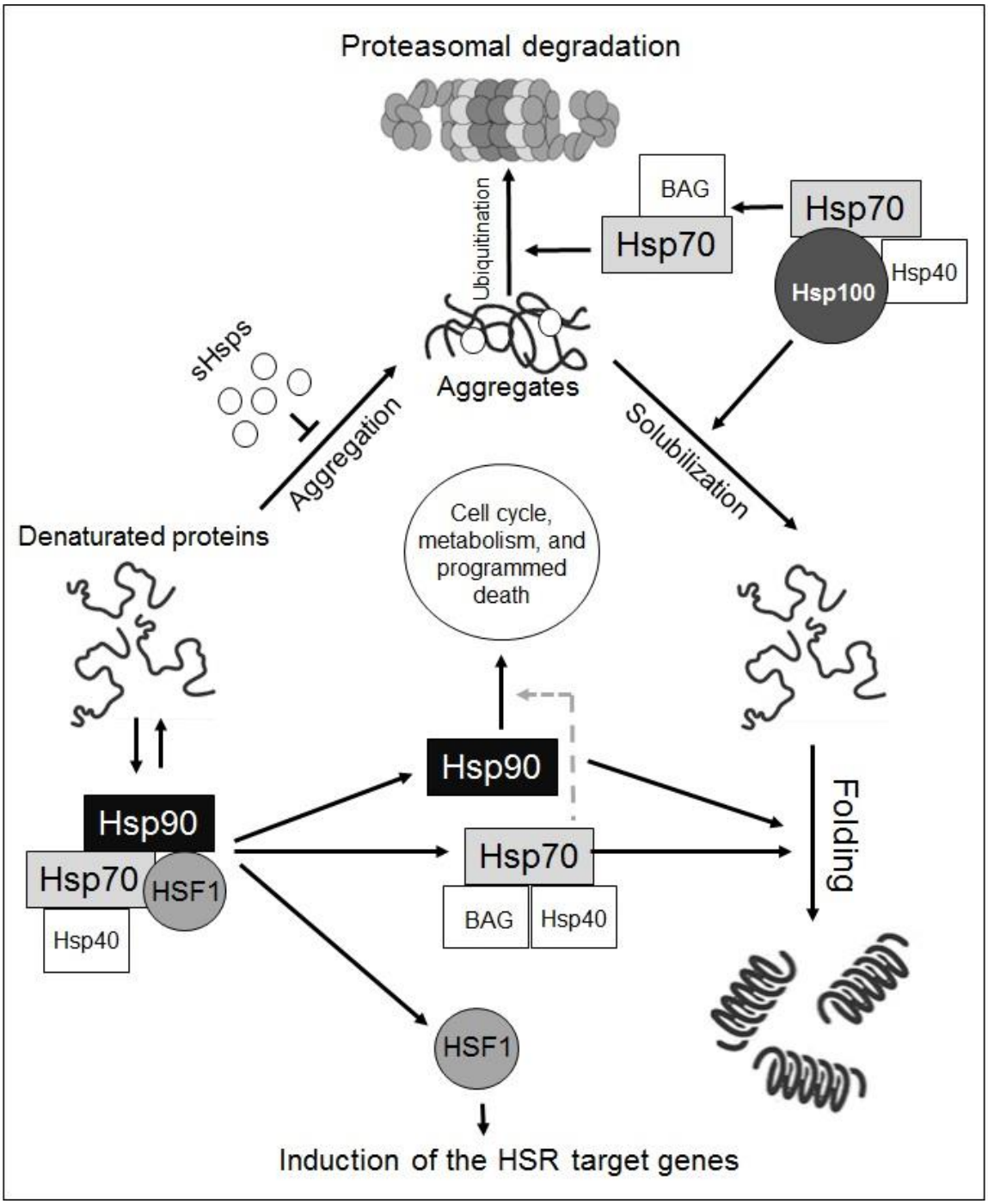

Figure 7. Simplified representation of heat shock proteins' functions in the eukaryotic protein quality system. In physiological conditions, HSF1 is sequestered in a cytosolic complex with Hsp90, Hsp70 and Hsp40. Releases of HSF1 in the presence of denatured proteins leads to induction of target genes, triggers the heat-shock response, and notably the synthesis of new heat shock protein. Hsp70 and Hsp90 are both parts of the peptides' folding machinery and also relay information about macromolecular integrity to other regulatory pathways. sHsps bind to denatured proteins to prevent their aggregation and precipitation and can be further sequestered in aggregates for easier resolution by Hsp100 complexes afterward. Switch between refolding and degradation is mainly coordinated with the help of co-chaperones such as Hsp40 and BAG proteins. Created by integrating data from references 16, 19, and 40-62.

Osmolytes accumulation

Osmolytes are low molecular weight organic molecules. Their signature role is to manage cell volume under water-stress conditions that may include extremes of pressure, changes in extracellular osmotic conditions, and exposition to extreme temperatures[66]. In order to understand the implication of osmotic stress in a context of heat-damages, it must be considered that shifts of osmotic pressure occurring in biological fluids (because of water loss) cause changes in the composition of the intracellular medium. Molecules such as salts, proteins and metabolites concentrate, which alters the balance of electrostatic strengths behind the thermodynamic equilibrium dictating macromolecular stability. In turn, this generates proteotoxic and oxidative stresses, which are the root cause for triggering the HSR.

In essence, there are three major chemical classes of osmolytes that protect organisms: (I) polyols and sugars, (I) methyl amines, and (III) amino acids[66,67]. In some organisms, they accumulate in the intracellular environment at relatively high concentrations, thereby increasing thermodynamic stability of folded proteins without perturbing other cellular processes[68]. In the yeast, for example, trehalose increases protein stability to high temperatures and reduces their aggregation[69-71]. Through balancing osmotic pressures with extracellular mediums, osmolytes can prevent intracellular water loss[72]. They can keep proteins soluble and thus prevent their aggregation despite changes in osmotic conditions, allowing in most extreme cases impressive cellular survival capabilities to dehydration, as occurs in tardigrades or resurrection plants[73,74]. Finally, they can offer some level of protection against oxidative stress damages[75,76], likely through a buffering effect. Another role of osmolytes worth mentioning in poikilotherms organisms (*i.e* that cannot regulate their own internal temperature) is protection against cold injuries[72]. Like a classic antifreeze, polyols reduce the supercooling point of water and thus allow cells to survive temperatures well below 0°C. Accumulation of osmolytes such as proline, glycogen, trehalose, myo-inositol and sorbitol were documented in terrestrial overwintering arthropods and plants[75-77].

In conclusion, intracellular osmolytes accumulation can directly enhance protein stability to above or suboptimal temperatures, although there is little literature supporting such properties in animal models *in vivo* so far. They also seem to offer some level of protection against potential side complications linked with thermal-stress, such as preventing osmotic water loss and protein aggregation in hyperosmotic medium (as would be the intracellular medium in dehydrated cells), as well as buffering oxidative damages. In a context of heat-tolerance in animals, accumulation of osmolytes could therefore be potentially relevant as complementary mechanisms for cells to enhance survival to transient deleterious conditions.

Controlling membrane fluidity

The issues caused by thermal stress for biological membranes are challenging (see Box 3). It has even been further argued that in cellular organisms, plasma membranes might be the primary sites of thermal wounding[12,72]. Disruption of the cell membrane indeed sets in motion a cascade of events involving the inactivation of membrane ATP-ase such as sodium–potassium pumps that maintain membrane potential in cells. In turn, the loss of cells' membrane potential causes, among other things, breakdowns in cell metabolism, loss of homeostasis and lethal inactivation of the nervous system in animals. While the causes of death due to heat-shock are multifactorial, it is nonetheless clear that maintaining membrane integrity, fluidity and permeability despite heat-stress is critical for organisms' survival[72, 78,79].

Exposition to cold decreases the membrane fluidity (membrane rigidification; Fig.5), which can be compensated by adapting the membrane content in unsaturated fatty acids. Alternatively, heat fluidizes membranes. This is compensated by the replacement of unsaturated fatty acids by saturated ones[79]. Living organisms are usually capable of synthesizing saturated fatty acids that are desaturated if needs be[80]. Modulation of fatty acid saturation is not the only mechanism for controlling membrane fluidity. Adapting the length of fatty acid as well as the ratio between lipids, proteins and cholesterol embedded in the membrane are complimentary means by which fluidity can be controlled (longer fatty acids, more proteins, and more sterols, reduce membrane fluidity through steric hindrance)[79-83].

In conclusion, in a context of heat-tolerance in animals, cells need to rigidify both their cell and organelle membranes to maintain proper homeostasis. This can be achieved by elongating fatty-acid chains, synthetizing *de novo* saturated phospholipids and sterols, and embedding more cholesterol and proteins inside the phospholipid bilayer. All those will result in reduced molecular movements inside membranes, allowing cells to lower their membrane fluidity, permeability, to maintain homeostasis and ultimately, to enhance their survival to above optimal temperatures.

The antioxidant response: keeping ROS damages in line

Reactive oxygen species (ROS) are highly reactive chemical species containing oxygen, such as hydrogen peroxide (H_2O_2) or hydroxyl radical ($^{\bullet}OH$). Being chemically aggressive as they are, they can potentially react with cellular structures and cause toxicity (Fig.3). At the molecular level, ROS production is intimately linked with the aerobic metabolism. The first report that the aerobic ATP chain production endogenously produced ROS came in 1966[84], followed by further work showing that isolated mitochondria indeed produced low levels of hydrogen peroxide[85]. Since then, mitochondria have been identified as an important source of ROS within eukaryotic cells[86]. During the mitochondrial oxidative phosphorylation process, reduction of molecular oxygen (O_2) mostly produces harmless water. However, it can also secondarily produce dangerously reactive intermediate anions such as superoxides ($^{\bullet}O^{-}_2$; Equation 1)[87] which are the precursor of most other ROS[18]. Dismutation of superoxides produce hydrogen peroxide (Equation 2). In turn, hydrogen peroxide may be reduced to hydroxyl radicals (Equation 3) or fully reduced to water (Equation 4). Please see Murphy *et al.*, 2009[86] for a more comprehensive account of reactions leading to the production of ROS.

(1) $O_2 + e^- \rightarrow {}^{\bullet}O^{-}_2$
(2) $2\ H^+ + 2{}^{\bullet}O^{-}_2 \rightarrow H_2O_2 + O_2$
(3) $H_2O_2 + e^- \rightarrow HO^- + {}^{\bullet}OH$
(4) $2\ H^+ + 2\ e^- + H_2O_2 \rightarrow 2\ H_2O$

Uncontrolled increase of ROS concentrations leads to free radical mediated chain reactions which indiscriminately target other macromolecules such as proteins, lipids, polysaccharides and DNA, impairing their structure and biological roles[15]. The shift in balance between oxidant/antioxidant in favor of oxidants, leading to macromolecular damages, is termed as "oxidative stress"[88]. Numerous evidences have linked oxidative stress to various pathologies[88], regulation of cell death[90], and aging[91], surrounding ROS with a huge amount of attention and literature. In a context of heat-stress in animal cells, it has been shown that the rate of production in the mitochondria of the superoxide anion $^{\bullet}O^{-}_{2}$ is increased[92], which likely lead to the subsequent production of other ROS. Perhaps thermal denaturation of REDOX proteins inside the mitochondria[93], or thermal denaturation of metalloproteins such as ferritin which releases transition metal ions capable of catalyzing REDOX reactions[94], are accountable for such effect. Overall, overproduction of ROS generates a great deal of potentially lethal oxidative stress in the cell, and especially in the mitochondria[15]. Damaged mitochondria are recycled through autophagy (see dedicated sub-chapter: *Modifying cellular death thresholds: autophagic and apoptotic pathways*). This reduces their numbers, reduces ATP production, and overall causes difficulties for the cell to both maintain its normal functioning and efficiently recover from heat-shock[15-18].

Cells thus respond to oxidative stress with antioxidant mechanisms. Through details of those mechanisms varies from species to species, molecules involved share universal features and can be segregated into two types: non-enzymatic antioxidants and enzymatic antioxidants. Non-enzymatic antioxidants are generally small organic molecules, chemically sensitive to oxidation, whose role will be to intercept free radicals and react with ROS at an efficient rate, conferring a buffer-protective effect against oxidative stress[95]. Few examples of the non-enzymatic antioxidant scavengers are vitamin C, vitamin E, polyphenols, carotenoids, and glutathione[96]. Enzymatic antioxidants are, as their name indicates, enzymes aimed at breaking down and removing free radicals. Important antioxidant enzymes include superoxide dismutases (SOD, which catalyze the reduction of $\bullet O^{-}_{2}$ into H_2O_2), catalases (which catalyze the reduction of H_2O_2 into water), peroxyredoxins (which catalyze the reduction of H_2O_2), thioredoxins (that catalyze the reduction of unwanted disulfide bonds between two cysteine residues in oxidized proteins), and finally enzymes in the glutathione system. Please see Birben *et al.*, 2012[88] for a detailed overview of antioxidant molecules and their mechanisms of action.

Finally, because elevated concentration of ROS leads to proteotoxic stress, there is evident crosstalk between pathways controlling the antioxidant response and the induction of Hsps[97-100]. In mitochondria, Hsps induction and antioxidant mechanisms are both necessary to prevent oxidative damages[99], damages further suggested to be another major source of thermal wounding in animals[15].

In conclusion, hyperthermia increases the production of mitochondrial ROS that can damage cellular structures. To counter those adverse effects, the antioxidant response involves non-enzymatic and enzymatic actors aimed at intercepting and breaking down free radicals. The antioxidant response complements molecular chaperones in buffering and repairing heat-induced damages, especially in the mitochondria. Consequently, both responses are essential to tolerate exposition to above-optimal thermal regimes.

Modifying cellular death thresholds: autophagic and apoptotic pathways

In higher eukaryotes, information about the integrity of DNA, proteins, cellular membranes and other important macromolecules integrates into pathways promoting cell survival or signaling programed cell death. While the existence of "controlled" form of death in cells may seem surprising at first, it has evolved as an important part of organisms' homeostasis and health[101]. Controlled forms of cell death mostly occur through orderly cellular fragmentation that facilitates the recycling of cellular component by the surrounding tissue[101]. It is a normal process in embryogenesis, cell turnover, it keeps fit and healthy cells in tissues and holds consequences on the organisms' ability to survive and recover various environmental constraints, including heat-stress[101-103].

Two main types of cellular deaths and one linked mechanism promoting cell survival will be discussed hereafter (other variants exist but will not be considered in the present manuscript): (1) necrosis, (2) apoptosis, and (3) autophagy (Fig. 8)[101-104].

- Necrosis is the most detrimental. It's a chaotic rupture of the cell's integrity caused by an array of damages that were either too fast for the cell to be able to adapt to, or too important to be monitored. Cellular components are anarchically released in the surrounding tissues, causing severe inflammation, offering ideal thriving conditions for microorganisms, and causing further complication for tissues to survive and recover[104]. The two other types of cellular deaths, autophagy and apoptosis, are controlled mechanisms that both initially root from feedback pathways detecting issues with macromolecular integrity and oxidative stress[105,106].
- Apoptosis occurs beyond a certain threshold of damages when feedback mechanism dictates that trying to promote cell survival would either be pointless or could be dangerous for the organism's health (for example, promoting the survival of cells with extended DNA damages can lead to pathologies)[105]. Thus, beyond a set point of damages, the cell undergoes a programed death implying a staged fragmentation with aim to facilitate the recycling of its components and avoid the deleterious effects of necrosis[101].
- Autophagy is a self-digestive act (autolysis) that, in a context of proteotoxic stress, occurs to recycle overly damaged structures (such as whole organelles and large toxic aggregates of denatured proteins) that would be too large to digest for smaller proteolytic system[102,103]. While this phenomenon can sometimes lead to cell death in extreme forms, it is primarily aimed at promoting cell survival[106,107], and represents a distinct yet complementary system to the HSR for cellular protein quality control[106]. Autophagy has further been linked with stress-tolerance in various organisms, highlighting its role in maintenance of proteostasis and recycling of damaged cellular components[106-110].

It is important to stress that signals of oxidative and macromolecular damages will either trigger autophagy or apoptosis depending on their severity. Both autophagy and apoptosis are tightly linked at their regulatory level, and their occurrence will rely on the balance between cellular damages caused by stresses, the repair and degradation mechanisms deployed by the cell (such as Hsps, antioxidants and autophagy), and the onset of apoptotic threshold[110]. Autophagic and apoptotic pathways thus rely on the complicated crosstalk between proteostasis, oxidative-stress, DNA and mitochondrial integrity, metabolic states and cell division, which itself is partially relayed by redox sensors and heat-shock proteins[15, 55-58, 98-106].

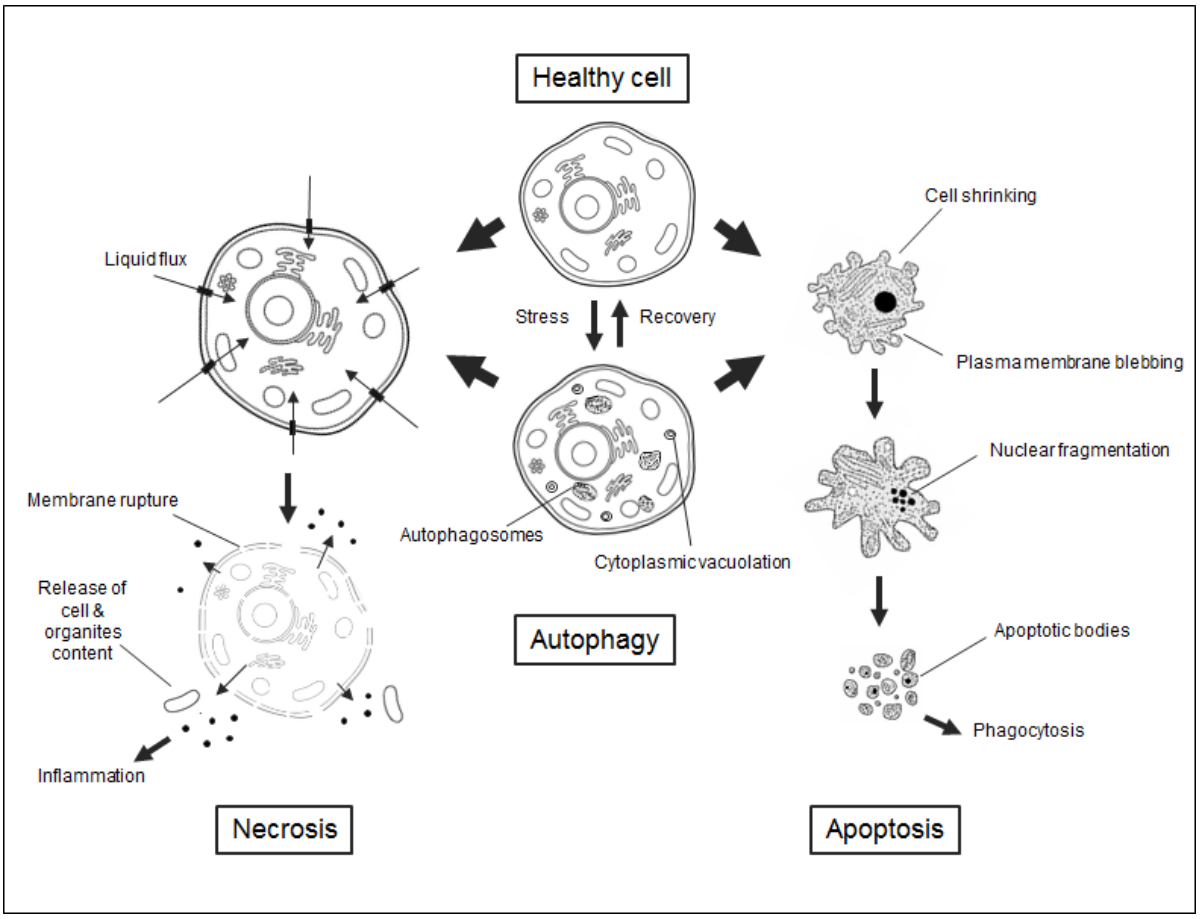

Figure 8. Simplified representation of two main forms of cell death, necrosis and apoptosis, and their link with autophagy. Necrosis implies cell membrane rupture and release of cells' content into the surrounding medium. Autophagy aims at promoting cell survival through autolysis. Finally, apoptosis is a controlled and orderly cell fragmentation leading to the formation of apoptotic bodies that will be recycled by the surrounding tissues.

In short, a simplified understanding of the implication of autophagy and apoptosis in a context of heat-stress tolerance is to conceive their flux pathways as final integrators of stress signals, either promoting survival through autolysis and self-recycling (autophagy) or switching to promote programed cell death (apoptosis), depending on the extent of the damages detected by the cell. Autophagic capability is one of the last lines of defense against proteotoxic stress and has been underpinned as central for heat-stress tolerance and recovery.

Conclusion

Shifts in molecular agitation oppose real challenges to cellular life, with ranging implications for key cellular structures and processes. A wide array of responses has evolved to stabilize and buffer damages that can result from exposition to non-optimal temperatures. The heat-shock response, over-expression of molecular chaperones and proteolytic systems maintains proteostasis. Active regulation of biological membranes composition ensures a proper fluidity. In some cases, accumulation of osmolytes keeps proteins soluble despite variation of osmotic pressure, and the antioxidant response counter heat-induce oxidative damages. Those responses and their feedback further integrate with those controlling cell autolysis, division and death to provide a tightly regulated, multifactorial answer to best cope with deleterious conditions cells are exposed to. The ability of cellular organisms to tolerate heat-stress heavily depends on these mechanisms and their modulation, whose peculiarities reflects the evolutive history of the considered organism. They have evolved to allow life to thrive regardless of the changing conditions encountered in most biomes and are to this day hotspot of research in cellular biology.

References

1. Ruiz-Mirazo, K., Peretó, J., & Moreno, A. (2004). A universal definition of life: autonomy and open-ended evolution. *Origins of Life and Evolution of the Biosphere*, *34*(3), 323-346.
2. Sparrow, E. M. (2018). *Radiation heat transfer*. Routledge.
3. Kondepudi, D. (2008). *Introduction to modern thermodynamics*. Wiley.
4. Van Holde, K. E., Johnson, W. C., & Ho, P. S. (2006). Principles of physical biochemistry.
5. Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual review of physiology*, *61*(1), 243-282.
6. Parsell, D. A., & Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual review of genetics*, *27*(1), 437-496.
7. Israelachvili, J. N. (2011). *Intermolecular and surface forces*. Academic press.
8. Stone, A. (2013). *The theory of intermolecular forces*. OUP Oxford.
9. Lapanje, S. (1978). *Physicochemical aspects of protein denaturation*. Wiley.
10. Hightower, L. E. (1991). Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell*, *66*(2), 191-197.
11. Heimburg, T. (2008). *Thermal biophysics of membranes*. John Wiley & Sons.
12. Konings, A. W. T., & Ruifrok, A. C. C. (1985). Role of membrane lipids and membrane fluidity in thermosensitivity and thermotolerance of mammalian cells. *Radiation research*, *102*(1), 86-98.
13. Lande, M. B., Donovan, J. M., & Zeidel, M. L. (1995). The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *The Journal of general physiology*, *106*(1), 67-84.
14. Gutteridge, J. M. (1995). Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical chemistry*, *41*(12), 1819-1828.
15. Belhadj Slimen, I., Najar, T., Ghram, A., Dabbebi, H., Ben Mrad, M., & Abdrabbah, M. (2014). Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. *International journal of hyperthermia*, *30*(7), 513-523.
16. Richter, K., Haslbeck, M., & Buchner, J. (2010). The heat shock response: life on the verge of death. *Molecular cell*, *40*(2), 253-266.
17. Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, *55*, 373-399.
18. Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of physiology*, *552*(2), 335-344.
19. Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature*, *475*(7356), 324.
20. Los, D. A., & Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1666*(1), 142-157.
21. Fineblum, W. L., & Rausher, M. D. (1995). Tradeoff between resistance and tolerance to herbivore damage in a morning glory. *Nature*, *377*(6549), 517.
22. Stetter, K. O. (1999). Extremophiles and their adaptation to hot environments. *FEBS letters*, *452*(1-2), 22-25.
23. Demirjian, D. C., Morís-Varas, F., & Cassidy, C. S. (2001). Enzymes from extremophiles. *Current opinion in chemical biology*, *5*(2), 144-151.
24. van de Vossenberg, J. L., Driessen, A. J., & Konings, W. N. (1998). The essence of being extremophilic: the role of the unique archaeal membrane lipids. *Extremophiles*, *2*(3), 163-170.
25. Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W., & Stetter, K. O. (1997). Pyrolobus fumarii, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 C. *Extremophiles*, *1*(1), 14-21.
26. Pysz, M. A., Ward, D. E., Shockley, K. R., Montero, C. I., Conners, S. B., Johnson, M. R., & Kelly, R. M. (2004). Transcriptional analysis of dynamic heat-shock response by the hyperthermophilic bacterium Thermotoga maritima. *Extremophiles*, *8*(3), 209-217.
27. Sørensen, J. G., Kristensen, T. N., & Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, *6*(11), 1025-1037.
28. Santoro, M. M., Liu, Y., Khan, S. M., Hou, L. X., & Bolen, D. W. (1992). Increased thermal stability of proteins in the presence of naturally occurring osmolytes. *Biochemistry*, *31*(23), 5278-5283
29. Murata, N., & Los, D. A. (1997). Membrane fluidity and temperature perception. *Plant Physiology*, *115*(3), 875.
30. Yu, B. P. (1994). Cellular defenses against damage from reactive oxygen species. *Physiological reviews*, *74*(1), 139-162.

31. Maiuri, M. C., Zalckvar, E., Kimchi, A., & Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nature reviews Molecular cell biology*, *8*(9), 741.
32. Evgen'Ev, M. B., Garbuz, D. G., & Zatsepina, O. G. (2014). *Heat shock proteins and whole body adaptation to extreme environments*. Springer.
33. Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in Drosophila. *Experientia*, *18*(12), 571-573.
34. Lindquist, S., & Craig, E. A. (1988). The heat-shock proteins. *Annual review of genetics*, *22*(1), 631-677.
35. Bence, N. F., Sampat, R. M., & Kopito, R. R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*, *292*(5521), 1552-1555.
36. Bozaykut, P., Ozer, N. K., & Karademir, B. (2014). Regulation of protein turnover by heat shock proteins. *Free Radical Biology and Medicine*, *77*, 195-209.
37. Arya, R., Mallik, M., & Lakhotia, S. C. (2007). Heat shock genes—integrating cell survival and death. *Journal of biosciences*, *32*(3), 595-610.
38. Lindquist, S. (1986). The heat-shock response. *Annual review of biochemistry*, *55*(1), 1151-1191.
39. Nguyen, A. D., Gotelli, N. J., & Cahan, S. H. (2016). The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC evolutionary biology*, *16*(1), 15.
40. Kregel, K. C. (2002). Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of applied physiology*, *92*(5), 2177-2186.
41. Mogk, A., Deuerling, E., Vorderwülbecke, S., Vierling, E., & Bukau, B. (2003). Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Molecular microbiology*, *50*(2), 585-595.
42. Mayer, M. P. (2010). Gymnastics of molecular chaperones. *Molecular cell*, *39*(3), 321-331.
43. Alderson, T. R., Kim, J. H., & Markley, J. L. (2016). Dynamical structures of Hsp70 and Hsp70-Hsp40 complexes. *Structure*, *24*(7), 1014-1030.
44. Takayama, S., & Reed, J. C. (2001). Molecular chaperone targeting and regulation by BAG family proteins. *Nature cell biology*, *3*(10), E237.
45. Fan, C. Y., Lee, S., & Cyr, D. M. (2003). Mechanisms for regulation of Hsp70 function by Hsp40. *Cell stress & chaperones*, *8*(4), 309-316.
46. Lüders, J., Demand, J., & Höhfeld, J. (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *Journal of Biological Chemistry*, *275*(7), 4613-4617.
47. Miller, S. B., Ho, C. T., Winkler, J., Khokhrina, M., Neuner, A., Mohamed, M. Y., ... & Mogk, A. (2015). Compartment-specific aggregases direct distinct nuclear and cytoplasmic aggregate deposition. *The EMBO journal*, *34*(6), 778-797.
48. Song, J., Takeda, M., & Morimoto, R. I. (2001). Bag1–Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. *Nature cell biology*, *3*(3), 276.
49. Pratt, W. B., & Toft, D. O. (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Experimental biology and medicine*, *228*(2), 111-133.
50. Ran, R., Lu, A., Zhang, L., Tang, Y., Zhu, H., Xu, H., ... & Sharp, F. R. (2004). Hsp70 promotes TNF-mediated apoptosis by binding IKKγ and impairing NF-κB survival signaling. *Genes & development*, *18*(12), 1466-1481.
51. Arndt, V., Dick, N., Tawo, R., Dreiseidler, M., Wenzel, D., Hesse, M., ... & Hoch, M. (2010). Chaperone-assisted selective autophagy is essential for muscle maintenance. *Current Biology*, *20*(2), 143-148.
52. Horváth, I., & Vígh, L. (2010). Cell biology: Stability in times of stress. *Nature*, *463*(7280), 436.
53. Gupta, R. S. (1995). Evolution of the chaperonin families (HSP60, HSP 10 and TCP-1) of proteins and the origin of eukaryotic cells. *Molecular microbiology*, *15*(1), 1-11.
54. Martin, J., Horwich, A. L., & Hartl, F. U. (1992). Prevention of protein denaturation under heat stress by the chaperonin Hsp60. *Science*, *258*(5084), 995-998.
55. Taipale, M., Jarosz, D. F., & Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature reviews Molecular cell biology*, *11*(7), 515.
56. Whitesell, L., & Lindquist, S. L. (2004). HSP90 and the chaperoning of cancer. *Nature Reviews Cancer*, *5*(10), 761.
57. Burrows, F., Zhang, H., & Kamal, A. (2004). Hsp90 activation and cell cycle regulation. *Cell Cycle*, *3*(12), 1530-1536.
58. Pratt, W. B. (1998). The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. *Proceedings of the Society for Experimental Biology and Medicine*, *217*(4), 420-434.

59. Zou, J., Guo, Y., Guettouche, T., Smith, D. F., & Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell*, *94*(4), 471-480
60. Haslbeck, M., Franzmann, T., Weinfurtner, D., & Buchner, J. (2005). Some like it hot: the structure and function of small heat-shock proteins. *Nature Structural and Molecular Biology*, *12*(10), 842
61. Kriehuber, T., Rattei, T., Weinmaier, T., Bepperling, A., Haslbeck, M., & Buchner, J. (2010). Independent evolution of the core domain and its flanking sequences in small heat shock proteins. *The FASEB Journal*, *24*(10), 3633-3642.
62. Haslbeck, M., Miess, A., Stromer, T., Walter, S., & Buchner, J. (2005). Disassembling protein aggregates in the yeast cytosol The cooperation of Hsp26 with Ssa1 and Hsp104. *Journal of Biological Chemistry*, *280*(25), 23861-23868.
63. Sugiyama, Y., Suzuki, A., Kishikawa, M., Akutsu, R., Hirose, T., Waye, M. M., ... & Ohno, S. (2000). Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. *Journal of Biological Chemistry*, *275*(2), 1095-1104.
64. Golenhofen, N., Der Perng, M., Quinlan, R. A., & Drenckhahn, D. (2000). Comparison of the small heat shock proteins αB-crystallin, MKBP, HSP25, HSP20, and cvHSP in heart and skeletal muscle. *Histochemistry and cell biology*, *122*(5), 415-425.
65. Wójtowicz, I., Jabłońska, J., Zmojdzian, M., Taghli-Lamallem, O., Renaud, Y., Junion, G., ... & Jagla, T. (2015). Drosophila small heat shock protein CryAB ensures structural integrity of developing muscles, and proper muscle and heart performance. *Development*, *142*(5), 994-1005.
66. Auton, M., Rösgen, J., Sinev, M., Holthauzen, L. M. F., & Bolen, D. W. (2011). Osmolyte effects on protein stability and solubility: a balancing act between backbone and side-chains. *Biophysical chemistry*, *159*(1), 90-99.
67. Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., & Somero, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science*, *217*(4566), 1214-1222.
68. Kumar, R. (2009). Role of naturally occurring osmolytes in protein folding and stability. *Archives of biochemistry and biophysics*, *491*(1-2), 1-6.
69. Singer, M. A., & Lindquist, S. (1998). Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose. *Trends in biotechnology*, *16*(11), 460-468.
70. Elbein, A. D., Pan, Y. T., Pastuszak, I., & Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. *Glycobiology*, *13*(4), 17R-27R.
71. Wiemken, A. (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek*, *58*(3), 209-217.
72. Denlinger, D. L. (2009). Cold/heat protection. In *Encyclopedia of Insects (Second Edition)* (pp. 179-183).
73. Hengherr, S., Heyer, A. G., Köhler, H. R., & Schill, R. O. (2008). Trehalose and anhydrobiosis in tardigrades–evidence for divergence in responses to dehydration. *The FEBS journal*, *275*(2), 281-288.
74. Bianchi, G., Gamba, A., Limiroli, R., Pozzi, N., Elster, R., Salamini, F., & Bartels, D. (1993). The unusual sugar composition in leaves of the resurrection plant Myrothamnus flabellifolia. *Physiologia Plantarum*, *87*(2), 223-226.
75. Bemani, M., Izadi, H., Mahdian, K., & Khani, A. (2012). Study on the physiology of diapause, cold hardiness and supercooling point of overwintering pupae of the pistachio fruit hull borer, Arimania comaroffi. *Journal of insect physiology*, *58*(7), 897-902.
76. Liang, X., Zhang, L., Natarajan, S. K., & Becker, D. F. (2013). Proline mechanisms of stress survival. *Antioxidants & redox signaling*, *19*(9), 998-1011.
77. Tanaka, K. (1995). Seasonal change in glycogen and inositol/sorbitol contents of the house spider, Achaearanea tepidariorum (Araneae: Theridiidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *110*(3), 539-545.
78. Los, D. A., & Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1666*(1), 142-157.
79. Los, D. A., Mironov, K. S., & Allakhverdiev, S. I. (2013). Regulatory role of membrane fluidity in gene expression and physiological functions. *Photosynthesis research*, *116*(2-3), 489-509.
80. Los, D. A., & Murata, N. (1998). Structure and expression of fatty acid desaturases. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *1394*(1), 3-15.
81. Cooper, R. A. (1978). Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells. *Journal of Cellular Biochemistry*, *8*(4), 413-430.
82. Espenshade, P. J., & Hughes, A. L. (2007). Regulation of sterol synthesis in eukaryotes. *Annual review of genetics*, *41*.
83. Chintalapati, S., Kiran, M. D., & Shivaji, S. (2004). Role of membrane lipid fatty acids in cold adaptation. *Cellular and molecular biology (Noisy-le-Grand, France)*, *50*(5), 631-642.

84. Jensen, P. K. (1966). Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles I. pH dependency and hydrogen peroxide formation. *Biochimica et Biophysica Acta (BBA)-Enzymology and Biological Oxidation*, *122*(2), 157-166
85. Boveris, A., & Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties andeffect of hyperbaric oxygen. *Biochemical Journal*, *134*(3), 707-716.
86. Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal*, *417*(1), 1-13.
87. Davies, K. J. (1995, November). Oxidative stress: the paradox of aerobic life. In *Biochemical Society Symposia* (Vol. 61, pp. 1-32). PORTLAND PRESS-LONDON.
88. Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal*, *5*(1), 9.
89. Wiseman, H., & Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochemical Journal*, *313*(Pt 1), 17.
90. Fleury, C., Mignotte, B., & Vayssière, J. L. (2002). Mitochondrial reactive oxygen species in cell death signaling. *Biochimie*, *84*(2-3), 131-141.
91. Stadtman, E. R., & Berlett, B. S. (1997). Reactive oxygen-mediated protein oxidation in aging and disease. *Chemical research in toxicology*, *10*(5), 485-494.
92. Mujahid, A., Sato, K., Akiba, Y., & Toyomizu, M. (2006). Acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, possibly via downregulation of uncoupling protein content. *Poultry science*, *85*(7), 1259-1265.
93. Messner, K. R., & Imlay, J. A. (1999). The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of Escherichia coli. *Journal of Biological Chemistry*, *274*(15), 10119-10128.
94. Mujahid, A., Sato, K., Akiba, Y., & Toyomizu, M. (2006). Acute heat stress stimulates mitochondrial superoxide production in broiler skeletal muscle, possibly via downregulation of uncoupling protein content. *Poultry science*, *85*(7), 1259-1265.
95. Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental physiology*, *82*(2), 291-295.
96. Nimse, S. B., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *Rsc Advances*, *5*(35), 27986-28006.
97. Cabiscol, E., Bellí, G., Tamarit, J., Echave, P., Herrero, E., & Ros, J. (2002). Mitochondrial Hsp60, resistance to oxidative stress, and the labile iron pool are closely connected in Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *277*(46), 44531-44538.
98. Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological reviews*, *82*(1), 47-95.
99. Katschinski, D. M., Boos, K., Schindler, S. G., & Fandrey, J. (2000). Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis. *Journal of Biological Chemistry*, *275*(28), 21094-21098.
100. D'Autréaux, B., & Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature reviews Molecular cell biology*, *8*(10), 813.
101. Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology*, *35*(4), 495-516.
102. Levine, B., & Yuan, J. (2005). Autophagy in cell death: an innocent convict?. *The Journal of clinical investigation*, *115*(10), 2679-2688.
103. Beere, H. M. (2004). The stress of dying': the role of heat shock proteins in the regulation of apoptosis. *Journal of cell science*, *117*(13), 2641-2651.
104. Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *The American journal of pathology*, *146*(1), 3
105. Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*, *5*(5), 415-418.
106. Dokladny, K., Myers, O. B., & Moseley, P. L. (2015). Heat shock response and autophagy—cooperation and control. *Autophagy*, *11*(2), 200-213.
107. Klionsky, D. J., & Codogno, P. (2013). The mechanism and physiological function of macroautophagy. *Journal of innate immunity*, *5*(5), 427-433.
108. Choi, A. M., Ryter, S. W., & Levine, B. (2013). Autophagy in human health and disease. *New England Journal of Medicine*, *368*(7), 651-662.
109. Juhász, G., Érdi, B., Sass, M., & Neufeld, T. P. (2007). Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes & development*, *21*(23), 3061-3066.
110. Kroemer, G., Mariño, G., & Levine, B. (2010). Autophagy and the integrated stress response. *Molecular cell*, *40*(2), 280-293.

Chapter I

Total Internal Reflection Accounts for the Bright Color of the Saharan Silver Ant

Quentin Willot*, Priscilla Simonis*, Jean-Pol Vigneron, Serge Aron
* Co-lead authors

Objective

This chapter investigates the origin of the silver sheen of the Saharan ant, *Cataglyphis bombycina*, as well as its potential role in thermoregulation. It allowed us to estimate the contribution of its dense array of hairs that reflects visible sunlight to the range of body temperatures workers naturally experience while foraging. By doing so, we could relate more precisely thermal equilibrium of workers to their physiological mechanisms of heat-tolerance in the following chapters II, III and IV.

PLOS ONE

RESEARCH ARTICLE

Total Internal Reflection Accounts for the Bright Color of the Saharan Silver Ant

Quentin Willot[1☯], Priscilla Simonis[2☯], Jean-Pol Vigneron[2†], Serge Aron[1*]

1 Evolutionary Biology & Ecology, Université Libre de Bruxelles, Brussels, Belgium, 2 Photonic of living Organisms Group, Research Center in Physics of Matter and Radiation (PMR), University of Namur, Namur, Belgium

† Deceased.
☯ These authors contributed equally to this work.
* saron@ulb.ac.be

Citation: Willot Q, Simonis P, Vigneron J-P, Aron S (2016) Total Internal Reflection Accounts for the Bright Color of the Saharan Silver Ant. PLoS ONE 11 (4): e0152325. doi:10.1371/journal.pone.0152325

Editor: Matthew Shawkey, University of Akron, UNITED STATES

Received: November 20, 2015 Accepted: March 11, 2016 Published: April 13, 2016

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by the Wallonia-Brussels Federation (PS), the Université de Namur (grant ARC2010-2015]033) (JPV), the Université Libre de Bruxelles (ARC2010-2015]5) (SA), the Belgian FRIA (QW), the FRS-FNRS (PS, SA), and the Belgian Fonds National de la recherche Scientifique (FRFC 2.4516.11) (SA). The authors acknowledge using resources from the Interuniversity Scientific Computing Facility located at the University of Namur (Belgium), which is supported by the FRS-FNRS under convention 2.4617.07. The funders had

Abstract

The Saharan silver ant *Cataglyphis bombycina* is one of the terrestrial living organisms best adapted to tolerate high temperatures. It has recently been shown that the hairs covering the ant's dorsal body part are responsible for its silvery appearance. The hairs have a triangular cross-section with two corrugated surfaces allowing a high optical reflection in the visible and near-infrared (NIR) range of the spectrum while maximizing heat emissivity in the mid-infrared (MIR). Those two effects account for remarkable thermoregulatory properties, enabling the ant to maintain a lower thermal steady state and to cope with the high temperature of its natural habitat. In this paper, we further investigate how geometrical optical and high reflection properties account for the bright silver color of *C. bombycina*. Using optical ray-tracing models and attenuated total reflection (ATR) experiments, we show that, for a large range of incidence angles, total internal reflection (TIR) conditions are satisfied on the basal face of each hair for light entering and exiting through its upper faces. The reflection properties of the hairs are further enhanced by the presence of the corrugated surface, giv- ing them an almost total specular reflectance for most incidence angles. We also show that hairs provide an almost 10-fold increase in light reflection, and we confirm experimentally that they are responsible for a lower internal body temperature under incident sunlight.

Overall, this study improves our understanding of the optical mechanisms responsible for the silver color of *C. bombycina* and the remarkable thermoregulatory properties of the hair coat covering the ant's body.

Introduction

Cataglyphis bombycina, the"silver ant" of the Sahara, the Sinai and the deserts of the Arabian Peninsula, is famous for its ability to withstand extremely high temperatures [1,2]. Workers come out from the nest during the hottest midday period, when temperatures exceed 50°C, to scavenge corpses of heat-stricken animals. By restricting foraging activity to the hottest period of the day, the ants minimize the chances of encountering their most frequent predator—a

no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

lizard that ceases all activities when the temperature becomes unbearable. This, however, requires that the ants themselves are effectively protected against excessive temperatures. Workers strictly limit the time of exposure to the light from the high sun and the heat radiated by the hot sand [2]. They are equipped with legs much longer than other ants relative to their body shape, allowing them to keep their body at a greater distance from the hot surface, and to run much faster reducing the duration of their sorties while maximizing cooling by convection [1,3,4]. In contrast to other organisms, workers produce heat-shock proteins before emerging from the nest, thereby avoiding a slow adaptation to the sudden heat exposure [5,6].

Recently, it has been shown that silver ants have evolved remarkable thermoregulatory solutions to cope with the thermally stressful conditions of their natural environment [7]. The dorsal side of the workers' head, thorax and abdomen is covered with a dense array of triangularly shaped hairs that are responsible for the ants silvery appearance and produce strong thermoregulatory effects (Fig 1). By measuring reflectance values in the visible and near-infrared (NIR) spectra, Shi et al [7] have shown that the presence of triangular hairs greatly enhances light reflection. Sunlight is presumably deflected by a combination of scattering within the tri- angular hairs (Mie scattering) and total internal reflection (TIR) on their bottom face. Based on radiative heat transfer experiments, these authors showed with thermal camera images that hairs allow the workers to maintain lower body temperatures. The latter are achieved by mini- mizing energy absorption in the visible and NIR ranges of the spectrum, and by maximizing heat emissivity in mid-infrared (MIR) where the blackbody radiation spectrum of the ant's body is at its maximum [7].

In this paper, we further investigate the geometrical optics underlying the high reflectivity of *C. bombycina* hairs. Using theoretical and empirical approaches, we demonstrate that the bright silver color of the ant *C. bombycina* stems from the TIR associated with the hairs that densely cover the ant body. First, we examined hair morphology using scanning and transmission electron microscopy. Second, using ray-tracing optical models, as well as bi-directional reflectance distribution function (BRDF) and attenuated total reflection (ATR) experiments, we provide evidence that the triangular shape of hairs allows TIR for a large range of incident angles. We also show that hairs are corrugated on their upper faces and that this enhances reflection properties, giving hairs an almost total specular reflectance beyond the critical angle at which TIR occurs, thus the silver color. Finally, we bring additional evidence on the effect of hairs as an adaptation to heat tolerance, by comparing reflectance and internal body heating rate between hairy workers and 'shaved' workers whose hairs were removed.

Materials and Methods

Samples of *C. bombycina* were collected in the sand dunes of Erg Chebbi, Merzouga, in Southeastern Morocco (3108'45"N, 0359'55"W). The dunes are public areas and the silver ant is not an endangered or protected species; no permission was required to collect the samples. To compare the reflectance and heating rate between hairy, untreated workers and 'shaved' work- ers whose hairs were removed, ants were anesthetized by CO_2 exposure and hairs covering the dorsal side of the abdomen were completely removed using a sharp scalpel blade

Electron microscopy analyses of hairs morphology

Samples for scanning electron microscopy (SEM) were prepared by first removing the legs from workers, and then gluing the head, thorax and abdomen with silver paint on a metallic sample holder. The whole mount was coated in metal (20 nm of gold) in order to ease charge elimination, and placed in a JEOL InTouchScope JSM-6010LV microscope. For transmission electron microscopy (TEM), samples were prepared by embedding a piece of cuticle with hairs

Fig 1. The silver ant *Cataglyphis bombycina*. Colonies contain a caste of spindly workers and a caste of soldiers with large heads and saber-shaped mandibles. (a) In full sunlight, workers and soldiers show a metallic sheen that justifies the vernacular name of the species. Photo copyright: P. Landmann. (b,c) The hairs covering the dorsal side of the workers' head, thorax (b) and abdomen (c) follow the cuticle's curvatures.

doi:10.1371/journal.pone.0152325.g001

in an epoxy resin infiltrating the structure at a constant temperature of 35°C for 48 hours, and hardened at 60°C for 72 hours. Then, 90 nm-slices were cut perpendicular to the hairs' axis and examined with a FEI Tecnai transmission electron microscope.

Experimentally testing total internal reflection

To experimentally test the TIR model, we first measured the direction and spectral radiance of 2 mm^2 of the abdomen of a *C. bombycina* worker using a viewing angle measurement system (Eldim EZContrast L80) that gives the full spectral bi-directional reflectance distribution func- tion (BRDF). The detector gives accurate data for emissions ranging from 400 nm to 700 nm. This method allowed us to obtain a full BRDF image for each wavelength measured. In order to obtain reflectance values, spectral radiance was normalized with radiance of incident light. Reflectance of hairy workers was compared with that of shaved workers. Data were obtained for 3 individuals in each group; no significant intra-group difference was observed. Second, we experimentally tested attenuated total reflection (ATR) by placing hairs on a selectively absorbing surface, thereby greatly reducing TIR. Under ATR conditions, the light is transmit- ted rather than internally reflected in the hair [8]. Hairs were deposited flat face down on a copper grid (support

for TEM samples) and examined with an optical microscope (Olympus BX61) in bright-field epi-illumination mode. The objective (x60) had a numerical aperture (0.85) large enough to illuminate a single hair under angles > 34.9°, which corresponds to con- ditions for observing TIR (see Results)

Ant heating rate

A 0.075mm diameter thermocouple (K-type Thermocouple chromel–alumel, RS Components Ltd) connected to a digital thermometer (Digital Thermometer 206–3738, RS Components Ltd) was inserted through a small incision in the rectum of a freshly killed ant. The same posi- tion under the first tergite was always reached. We did not witness any bleeding or liquid loss that might have been a source of evaporative cooling. We let the abdomens reach room temper- ature (21°C) prior to measurements. The dorsal surface of each abdomen was exposed perpen- dicular to the light emitted by a solar simulator (ORIEL Corporation USA, No. 81172), away from any possible interfering surfaces. The device uses a 1000 W xenon arc lamp (power sup- ply: ORIEL No. 68820). Wavelengths above 1100 nm were removed with a water filter to limit blackbody radiation measuring only the effect of visible and NIR radiation (ORIEL No. 61945). Temperature elevation of the abdomen was recorded every 5 sec for 90 sec to approximate tem- perature equilibrium. Measures were repeated 3 times per abdomen, on 7 hairy and 7 shaved individuals; only ants of same size were used. All experiments were performed under constant temperature in an air-conditioned room. Initial rate of heat gain K was estimated following [9], as $K = (T_{ex}{}^{m}0.5)/t_{1/2}$), with T_{ex} being the excess temperature (*i.e.*, the difference between ambi- ent and sample temperature) and $t_{1/2}$ corresponding to the heating half-time. Comparisons of mean temperatures between hairy and shaved *C. bombycina* were performed using unpaired *t*- tests, using *Graphad Prism6* software.

Results

Hair morphology

SEM analyses confirm that the hairs covering the cuticle assume a triangular cross-section (Fig 2a). Our detailed measurements reveal that they have a length of the order 200–300 μm, a width w = 3.5 μm and a height h = 2.4 μm. The angle at the top (± SD) is on average equal to 72 ± 2°, and the other angles (β) are both 54 ± 2° (n = 8). The two upper surfaces of each hair are corrugated by parallel grooves, while the bottom face (base) is not corrugated (Fig 2b). The corrugations on the upper sides are parallel 66 nm deep flutes separated from each other by 204 nm. The flutes run oblique to the longitudinal axis of the hairs (Fig 2c). TEM shows that the basal faces of the hairs are parallel to each other and to the cuticle surface of the ant (Fig 2d). The distance between the basal face and the cuticle surface varies from a few μm to 50 μm.

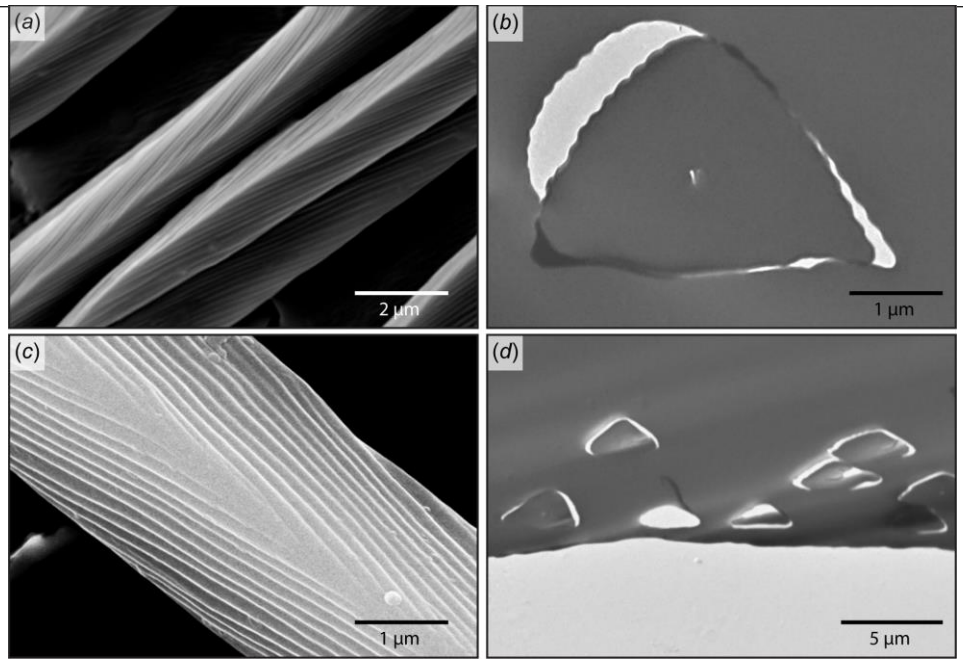

Fig 2. Electron microscopy images of the hairs covering the dorsal side of the body of *C. bombycina*. (a) Scanning electron microscopy (SEM) of the hairs covering the cuticle of ants. Hairs have a triangular shape and end in a sharp point. (b) Transmission electron microscopy (TEM) image of the cross-section of a hair. The two upper sides of the triangular shape are corrugated and the lower side (the triangle base) is flat. (c) Close up on the corrugations covering the two upper side of the triangular shape. (d) TEM image of hairs shows that they all adopt the same orientation, with their flat basal side parallel to each other and to the cuticle surface. The corrugated sides of the hairs are turned up as is clearly visible on image (a).

doi:10.1371/journal.pone.0152325.g002

Total internal reflection: prism model and experiments

Ray-tracing model. The relatively small size of the hairs compared to the light wavelength does not hinder us from using geometric optics to model the light path. Fig 3a gives a schematic representation of a hair, with a light ray entering through one of the upper faces, totally reflected on the smooth basal plane, and exiting through the opposite upper face. After a sequence of transmission, reflection and, again, transmission, the light continues in the inci- dent plane when neglecting weak reflections at the entrance and exit surfaces due to Mie scattering. Under these conditions, the incident and the emergence angles measured from the normal to the basal plane (*i.e*, the vertical) are equal (angle *i*, Fig 3a).The reflection on the basal plane of the hair is total if the local incidence angle $\beta + \varphi$ exceeds the critical angle θc given by

$$\sin\theta_c = \frac{n_{air}}{n_{chitin}} \quad (1)$$

Considering a refractive index of chitin $n_{chitin} = 1.56$ [10,11] and a refractive index of air $n_{air} = 1$, Eq (1) gives a critical angle on the basal plane $\theta c = 39.9°$. To reach this critical angle, the external incidence angle of the light on the hair is

$$i = \beta - \arcsin\left(\frac{n_{chitin}}{n_{air}}\sin(\beta - \theta_c)\right) \quad (2)$$

Eq (2) gives the critical external incidence angle $i = 31.7°$ with respect to the vertical, below which no TIR is possible. It should be noted, however, that the refractive index of the chitin is wavelength dependent [8,11–13] and directly affects the value of the critical external angle.

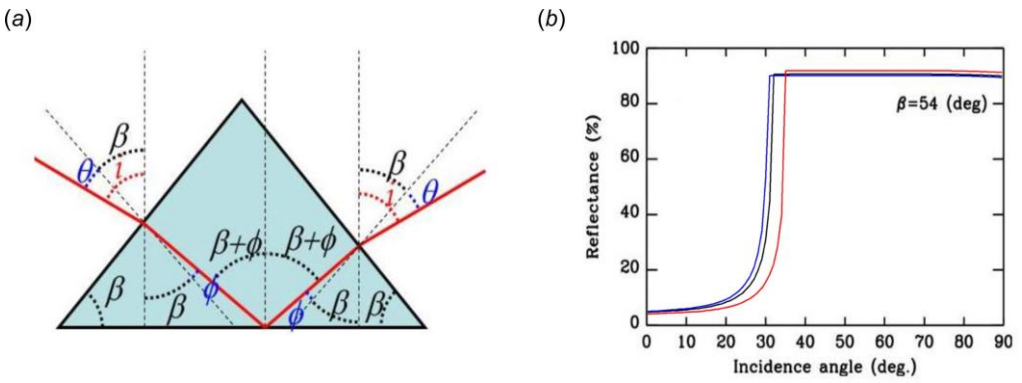

Fig 3. Total internal reflection prism model. (a) Ray-tracing model of TIR on the basal face of a triangular hair. The light enters through an upper face; it is totally reflected on the basal plane and exits through the opposite upper face. (b) Total reflectance of a hair (TIR and Mie scattering) as a function of the external incidence angle *i*, for an average chitin refractive index n_{chitin} = 1.56 (black), and for refractive indexes at 350 nm ($n_{chitin/350}$ = 1.58; blue) and 800 nm ($n_{chitin/800}$ = 1.51; red). For n_{chitin} = 1.56, the reflectance is low for sub-critical incidences $i < 31.7°$, then dramatically increases until i 34.9° at which angle total reflection occurs on the basal plane, parallel to the cuticle surface, at all visible wavelengths. A transfer-matrix model from wave optics results in a less steep transition from low to high reflectivity (data not shown).

doi:10.1371/journal.pone.0152325.g003

Due to weak variation of the chitin refractive index in the visible range, *i* increases from 30.3° to 34.9° as wavelengths of incident light increases from 350 to 800 nm. That is, for each single hair of *C. bombycina*, incidences angles *i* larger than 34.9° should result in a very intense reflec- tion at all visible wavelengths. This is illustrated in Fig 3b, which gives the calculated reflectance of a hair as a function of the external incidence angle *i*, for an average chitin refractive index of 1.56. In the high reflectance range, the overall reflection is not 100% because transmission through the upper faces of the prism is not perfect: a reflection in excess of about 5% removes intensity from the reflection on both the entry and exit faces. Still, more than 90% of the incident light is reflected. Thus, hairs are excellent mirrors for all external incidence angles $i > 34.9°$. Because reflection is directional for the whole visible wavelengths, this accounts for the silver sheen of the ant.

Influence of corrugations on reflectance. One noticeable feature of the hairs is the occurrence of corrugations on the entry and exit upper faces, contrasting with the flat basal face (Fig 2b). We explored the effect of such corrugations on reflectance. The spacing between two adjacent corrugations (b = 204 nm) is too short to produce diffraction. The effect of the corruga- tions is to slowly increase the effective refractive index when light enters the hairs, thereby acting as a kind of anti-reflection coating. This improves transmission of incident light through the upper surfaces of the hairs, and thereby increases the amount of light reflected by TIR.

To estimate the influence of corrugations on the light reflected by the entering face of a single hair, we compared the reflectance between a planar surface and a corrugated surface under normal incidence. For the planar surface, reflectance is calculated according to [14] as,

$$R = \left| \frac{n_{chitin} - n_{air}}{n_{chitin} + n_{air}} \right|^2 \quad (3)$$

The refractive index on the external side of the interface is n_{air} = 1. Taking into account that the refractive index of chitin depends on the wavelength of incident light, Eq (3) gives an average reflectance R = 5.4% (using a range of 350–800 nm) (Fig 4).

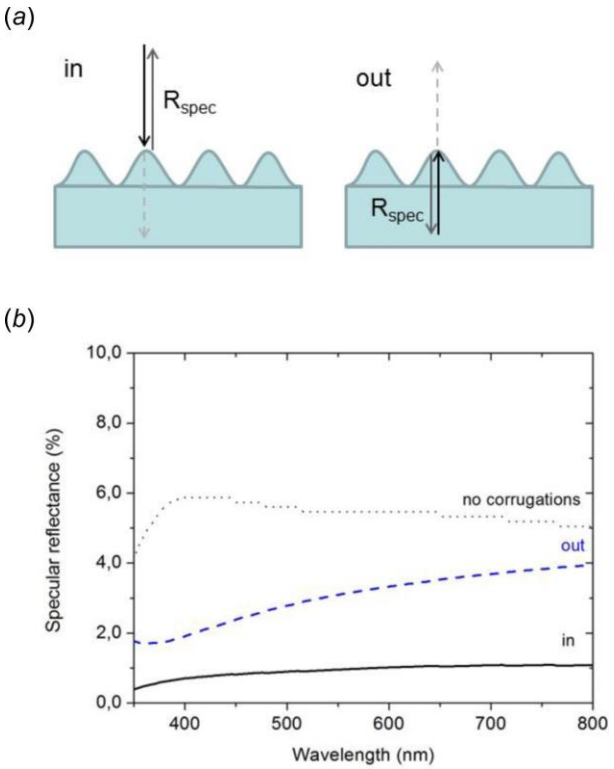

Fig 4. Influence of corrugations on the upper faces of the hairs on light reflection. (a) Schematic view of the corrugated chitin profile for light entering (in) and exiting (out) a hair. (b) Comparison of the specular reflectance between a flat chitin surface and a chitin surface corrugated by a sinusoidal profile, at normal incidence (see text). Corrugations significantly attenuate the reflection on both entry (in) and exit (out) of the light. This results in a global prism reflection ranging from 95% (800 nm) to 98% (350 nm) for incidence angles producing a total internal reflection. 64 monochromatic plane waves were used to reach the convergence of the transfer-matrix calculation.

doi:10.1371/journal.pone.0152325.g004

For the corrugated surface, we approximated the corrugation profile by a sinusoidal function with amplitude of 66 nm and a pitch of 204 nm, in line with experimental values (see above). Using a scattering-matrix approach [15], we calculated the specular reflectance (*i.e.*, incident angle = reflectance angle) of the sinusoidal profile. We considered a normal incidence for both the light entering and exit- ing the chitin. As shown Fig 4, corrugations considerably reduce the reflectance in the whole visible range. For the entering light, this reduction reaches a full order of magnitude, with a reflectance equal to R = 0.5%. Corrugations also reduce reflectance of exiting light, though to a lesser extent, with a specular reflectance varying from 2 to 4%. Altogether, these results show that corrugations on the upper faces of a hair have two major optical consequences on the scattering: they increase substantially the amount of light entering each hair and the amount of light exiting the hair after the TIR. Ultimately, this enhances TIR on the basal face of the hair, thus the reflectance of the ant.

Influence of abdomen curvature on reflectance. The ellipsoidal shape of the abdomen leads to a wide distribution of incident light directions, most being in a non-perpendicular plane to the longitudinal axis of the hairs. By using a Fourier-transform based spectrometer, Shi et al. (2015) [7] showed that hairs enhance reflectivity over all angles and that reflectivity becomes

particularly strong beyond 30°, which is consistent with our own TIR model pre- sented Fig 3.

To analyze the exact direction of the reflected light by the whole abdomen as a function of the incident angle of exposure, we summed the reflectance on a single hair over all incident directions [16]. In this situation, each incident direction is defined by a polar angle θ (*i.e.*, the latitude of the incident light) and an azimuthal angle φ (*i.e.*, the longitude of the incident light). Fig 5a gives the spectral radiance map on a hair for all incident directions. As shown, for all azimuthal angles TIR conditions are fulfilled for a wide range of polar angles. Consistent with the TIR conditions of the model (Fig 5a), spectral bi-directional reflectance distribution function (BRDF) analyses of the abdomen of *C. bombycina* workers show a ring of high reflectance for polar angles of approximately 30° to 60° (Fig 5b). For polar angles < 30° (*i.e.*, the center of the intensity map) almost no reflection is detected, which is in agreement with a light passing through the hairs without TIR due to small incidence angles. The dark, external ring observed on Fig 5b for polar angles between 50° and 80° may result from two causes. First, it may stem from the viewing angle of the measurement system and its relatively low collection efficiency (65%) for high polar angles [17]. Second, the many hairs that are oriented parallel to the incident ray do not reflect the light at polar angles > 60°, as shown Fig 5a for a single hair. This particular orientation results in the hairs acting as waveguides. Asymmetry of measured radiance on Fig 5b is probably due to hairs being aligned on the body of the ants (Fig 1c), hence the effect of only a subsample of incidence angles could be detected.

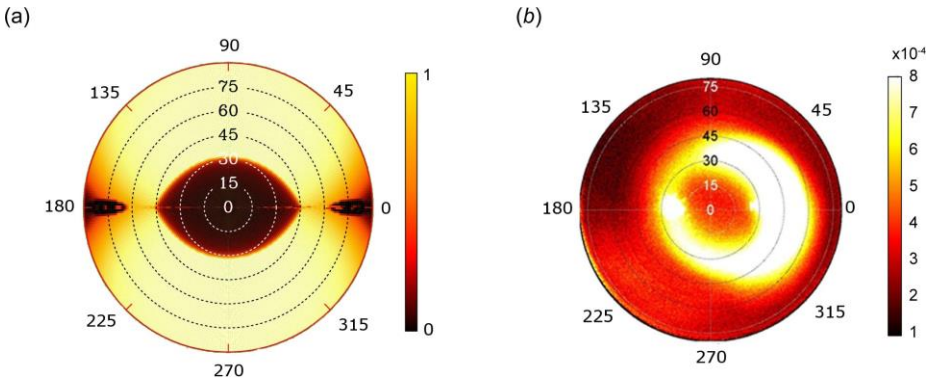

Fig 5. Spectral radiance maps of hairs in *C. bombycina*. Concentric circles correspond to the polar angles θ of the reflected light; counter-clockwise external angles are the azimuthal angles φ of the reflected light. (a) Calculation of the spectral radiance of a prism with triangular cross-section mimicking a single hair. The hair is oriented at an angle of φ = 0° and θ = 90° (azimuth 0–180 parallel to the axis of the hair). The reflection is total for all incidence angles, except at small incidence angles. (b) Experimental measure of the spectral radiance of a 2mm^2 surface of a worker abdomen covered with hairs. The hairs are oriented perpendicularly to the incident plane (azimuth 90–270 parallel to the axis of the hairs). Shown are data for an incident wavelength of 498 nm; similar patterns are obtained for other wavelengths (400–700 nm).

doi:10.1371/journal.pone.0152325.g005

Attenuated total reflection. Consistent with attenuated total reflection (ATR), TIR is dramatically reduced when hairs are placed on an absorbing surface of copper (Fig 6a). Highlights are observed on parts of the hair that overhang between wires, while parts in contact with the wire appear transparent so that the underlying copper surface can be seen. On the overhanging part, only the edges are bright; this is because TIR is effective on the basal plane only when light hits either side of the hair with an angle greater than 34.9° and/or when light enters the top of the hairs so that TIR occurs on the upper faces (Fig 6b).

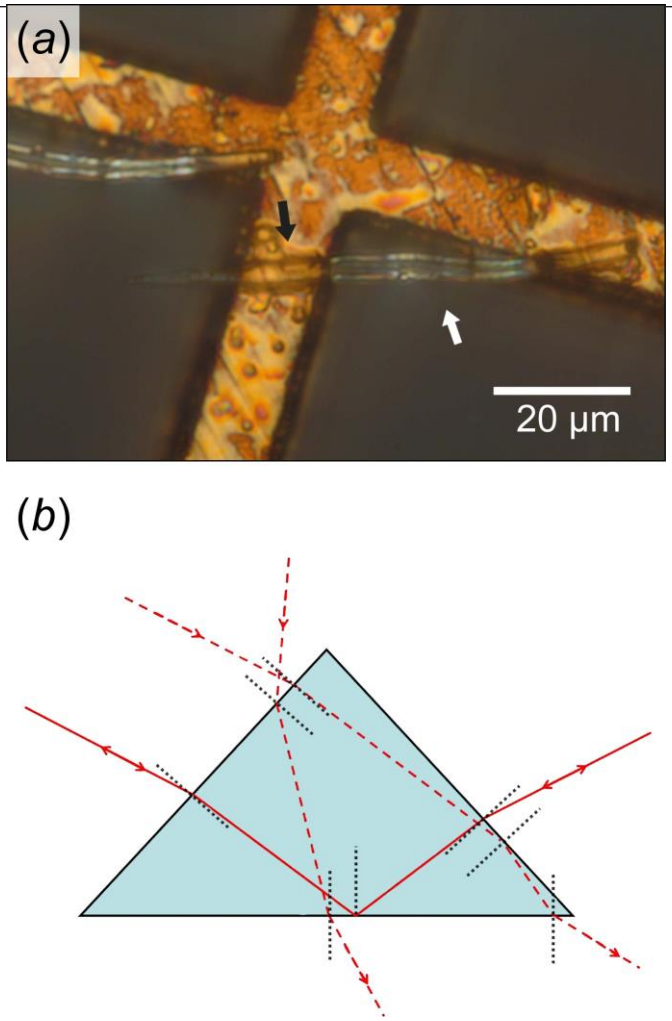

Fig 6. ATR experiment on ant hairs. The hairs are placed on an absorbing copper surface, with the bottom surface in contact with the copper. A part of each hair rests on the surface, whereas the other part is overhanging. Hairs are exposed to a spot of incident light large enough to hit both upper faces. (a) On the overhanging part of the hair, TIR occurs on the basal surface so that the reflected light is observed on both upper faces of the hair (as shown by the two bright lines bordering it). In contrast, for the part of the hair in contact with the copper wire, TIR is attenuated by the proximity of the selectively absorbing surface (the two bright lines bordering the hair disappear). (b) Schematic diagram showing TIR on the basal face of a triangular hair (plain line); under TIR conditions, no light can exit from the top portions of the two upper faces of the hair (dotted lines).

doi:10.1371/journal.pone.0152325.g006

Reflectance measurements. Comparison of the spectral radiance in the visible between hairy workers and shaved workers by BRDF measurements shows that the presence of hairs allows an almost constant 10-fold increase in radiance. As shown Fig 7a, mean reflectance between 425 and 700 nm is 0.61 for hairy ants and 0.07 for shaved ants. These results are consis- tent with Fig 3b showing very intense reflectance at all visible wavelengths for the given inci- dence (50°). They are also in line with [7] Fourier-transform based spectrometer measurements, showing a significant increase in light reflectance in the visible and near-infrared range of the spectrum on hair-covered regions of the body.

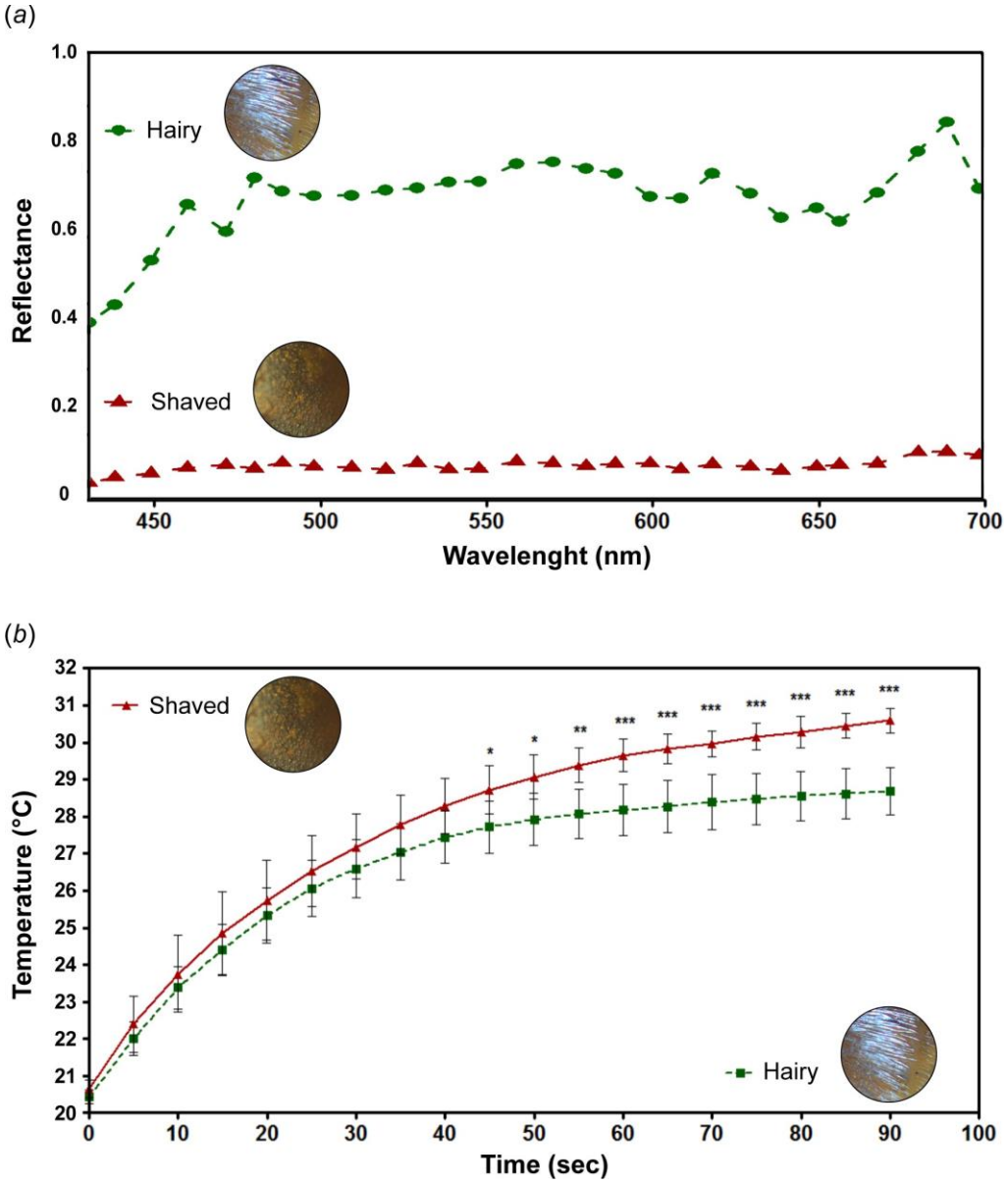

Fig 7. Reflectance and heating curves of hairy workers and shaved workers of *C. bombycina*. (a) Reflectance. TIR is induced on the ant abdomen with an incident light at 50° and azimuth 0. Reflectance is about 10-fold higher in the presence of hairs, for wavelengths ranging from 425 to 700 nm. (b) Heating curves. Abdomens are exposed for 90 seconds to the light emitted by a solar simulator whose visible spectral intensity is identical to the summer Saharan sun. Difference in excess temperature becomes significant after 45 seconds; after 90 seconds exposure, the difference in excess temperature between both conditions reaches 1.91°C. Unpaired Student *t*-test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

doi:10.1371/journal.pone.0152325.g007

Ant heating rate

We compared temperature elevation between shaved and hairy workers exposed to the light emitted by a solar simulator. The lamp intensity was calibrated according to the global irradi- ance of Saharan summer midday sun for a flat surface at sea level (Wilaya d'Adrar, Algeria; SMART95 software). Exposition to the solar simulator results in different heating curves between abdomens (Fig 7b). Averaged K values (± SD) are 0.27 ± 0.12 and 0.19 ± 0.03 for shaved and hairy abdomens, respectively (*t*-test, $p = 0.15$). Difference in excess temperature between shaved and hairy ants becomes significant after 45 sec exposition (1.00°C, $p = 0.026$); it is maximal after 90 seconds (1.91°C, $p < 0.0001$).

Discussion

Our results show that total internal reflection in the hairs of the Saharan ant *C. bombycina* is responsible for its bright silver sheen. Each hair has the shape of a prism with a triangular cross-section within which the conditions for TIR are met for a wide range of incidence angles. In addition, our simulations indicate that corrugations on the entrance and exit faces of the hairs further increase reflectance across the whole visible wavelength range. For incidence angles ranging from 34.9° to 90°, the reflectance is very close to 100% for all azimuthal angles. For smaller incidence angles, light can leak through a hair. However, the light transmitted will be reflected by other hairs underneath. Together, these physical properties account for a strong mirror effect in the whole visible range, hence, the silver color of the ant. Consistent with this, ATR experiments show that the proximity of a metallic surface attenuates TIR, suppressing the silver color.

Our data show that the hairs covering the ant dorsal face reflect up to 10 times more light than shaved cuticles over the visible spectrum (425–700 nm). The values of reflectance obtained in the present study are similar to those reported by Shi et al. [7]. These authors mea- sured reflectance values with a Fourier-transform based spectrometer allowing full visible and NIR spectral range, while our measures of radiance were performed by BRDF in the visible range only. The energy of direct sunlight is composed approximately of 40% visible and 50% NIR radiation (UV below 400 nm and IR beyond 1500 nm account for 2–4% and 6–8% of this energy, respectively;[18]). NIR radiation is therefore a major source of incoming energy that may greatly affect the thermal steady state of the ants. It was shown [7] that hairs also largely enhance NIR reflectance.

The thermoregulatory properties of the hairs coating stem from at least two effects [7]: (*i*)a reduction of solar energy intake by the mirror effect in the visible and NIR range of the spectra, which is further confirmed by our own results, and (*ii*) enhanced heat dissipation by radiation in the mid-IR where the black body radiation spectrum of the ant is at its maximum. By expos- ing individuals to a high-power xenon lamp to simulate the solar spectral distribution at the desert sand surface, these authors conducted an elegant set of thermodynamic experiments both in vacuum (to examine the effect of thermal radiation) and in air

(to study the interplay of thermal radiation and convection). Thermal camera images gave evidence that hairy workers maintain significantly lower body temperatures than those with the hairs removed. Our heating rate experiments, based on direct measures of temperature in the body of ants, corroborate these results. They show that hairy workers exposed to solar simulated light experience a reduc- tion in excess internal temperature up to 2°C compared with shaved workers. Even a small dif- ference in body temperature may prove highly ecologically relevant for *C. bombycina*, as it can make the difference between life and death for such a small insect foraging so close to its upper critical thermal limit. If such a hair coating is an efficient way to prevent overheating, why hasn't it evolved in more desert insects? Activity period of ectotherms is limited both by their lower and upper temperature tolerance. During the cooler Saharan season, dark-colored diur- nal ectotherms will be at advantage by absorbing more direct solar energy, thereby increasing their daily activity. Outside this period, they typically hide during the hottest hours of the day [19]. In contrast, foraging activity in *C. bombycina* is restricted to a narrow thermal window (48–51°C) delimited by heat stress (upper limit) and predatory pressure by their main lizard predator (*Acanthodactylus dumerili*) (lower limit) [2]. Thus, their lower limit of activity is far above physiological demands, whereas their upper limit depends on their ability to withstand heat. Consequently, unlike other desert insects, *C. bombycina* would gain in reflecting incident sunlight all year round.

So far, TIR has been reported as limiting light extraction in fireflies [20], and as a side effect in superhydrophobic leaves immersed in water [21]. Nature often uses other (more compli- cated) methods to produce a bright metallic coloration [14,22]. Bragg mirrors, for instance, have been described in other Hymenoptera. This kind of mirror uses light-wave interference in a one-dimensional layered structure to produce a high specular reflection, but often over a limited range of wavelengths. A reflectance covering a broader range of wavelengths

can be obtained with a stack of layers with graded-thickness, such as those encountered in some Scara- baeidae [23]. In beetles of the subfamily Rutelinae, the alternation of refractive index values in the multilayer is caused by the helical distribution of anisotropic chitin bars, in the form of a discrete Bouligand structure [24–26]. The large number of layers allows a highly efficient broadband reflectance that avoids iridescence. This structure is obviously much more complex than that found in the ant *C. bombycina*.

The triangular form of the hairs in the Saharan ant contrasts with the typical cylindrical shape [27,28] or flattened plate-like shape [29] of bristles documented in most arthropods. Depending on the species and their body position, the morphology of hairs has evolved to serve several functions including defense, locomotion, prey capture, pheromone dispersal, or temperature sensing [28,30,31]. The triangular shape that evolved in *C. bombycina* optimizes the backscattering of light and is responsible for the silver sheen of the ant. While reflection allows insects to gain less heat under direct sunlight [7,32], it could also serve other purposes such as camouflage [33] or communication [34]. Current studies indeed show that triangularly shaped hairs also occur in ground-dwelling ants from the rainforest, which are exposed to low insulation conditions (Simonis, unpubl. data). Therefore, several mechanisms

might have been jointly implicated in positive selection for the reflective coating in the silver ant.

Acknowledgments

This work was supported by the Belgian FRIA (QW), the FRS-FNRS (PS, SA), and the Univer- sité Libre de Bruxelles (ARC 2010–2015 #5; SA). The authors acknowledge using resources from the Interuniversity Scientific Computing Facility located at the University of Namur (Belgium), which is supported by the FRS-FNRS under convention 2.4617.07. We thank M. Oua- lim, Y. Bahou and M. Harmouchi for collecting samples, G. Ullens de Schooten for sample preparation, H. Darras for drawing the figures, D. Bolsée and the Belgian Spatial Aeronomy Institute (IASB) for their help with the solar simulator. S. Berthier, J.L. Deneubourg, B. Gass- ner, S. Massar, and J.M. Pasteels made helpful comments on a first draft of the manuscript. We also thank two anonymous reviewers for their comments and suggestions, which have substan- tially improved the manuscript.

Author Contributions

Conceived and designed the experiments: QW PS JPV SA. Performed the experiments: QW PS. Analyzed the data: QW PS SA. Contributed reagents/materials/analysis tools: PS SA. Wrote the paper: QW PS SA.

References

1. Délye G. Observations sur la fourmi saharienne *Cataglyphis bombycina* Rog. Insect Soc. 1957; 4: 72– 83.
2. Wehner R, Marsh AC, Wehner S. Desert ants on a thermal tightrope. Nature. 1992; 357: 586–7.
3. Marsh AC. Aspect of the ecology of Namib desert ants. M.Sc. Thesis, University of Cape Town. 1985.
4. Sommer S, Wehner R. Leg allometry in ants: extreme long-leggedness in thermophilic species. Arthro- pod Struct Dev. 2012; 41: 71–7. doi: 10.1016/j.asd.2011.08.002 PMID: 21992805
5. Gehring J, Wehner R. Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. P Natl Acad Sci USA. 1995; 92: 2994–8.
6. Moseley PL. Heat shock proteins and heat adaptation of the whole organism. J Appl Physiol. 1997; 83: 1413–7. PMID: 9375300
7. Shi NN, Tsai CC, Camino F, Bernard GD, Yu N, Wehner R. Keeping cool: Enhanced optical reflection and radiative heat dissipation in Saharan silver ants. Science. 2015; 349: 298–301. doi: 10.1126/ science.aab3564 PMID: 26089358
8. Harrick NJ. Internal Reflection Spectroscopy. New York: John Wiley & Sons Inc; 1967.
9. Willmer PG, Unwin DM. Field analyses of insect heat budgets: reflectance, size and heating rates. Oecologia. 1981; 50: 250–5.
10. Vukusic P, Sambles JR, Lawrence CR, Wootton RJ. Quantified interference and diffraction in single Morpho butterfly scales. P Roy Soc B-Biol Sci. 1999; 266: 1403–11.
11. Berthier S, Charron E, da Silva A. Determination of the cuticul index of the iridescent butterfly *Morpho menelaus*. Opt Commun. 2003; 228: 349–56.
12. Leertrouwer HL, Wilts BD, Stavenga DG. Refractive index and dispersion of butterfly chitin and bird ker- atin measured by polarizing interference microscopy. Opt Express. 2011; 19: 24061–66. doi: 10.1364/ OE.19.024061 PMID: 22109431
13. Stavenga DG, Leertrouwer HL, Wilts BD. Quantifying the refractive index dispersion of a pigmented bio- logical tissue, using Jamin-Lebedeff interference

microscopy. Light Sc Appl. 2013; 2: e100.

14. Vigneron JP, Simonis P. Structural colours. In: Casas J, Simpson SJ, editors. Advances in Insect Phys- iology. Burlington: Academic Press; 2010. p. 181–218.
15. Vigneron JP, Lousse V. Variation of a photonic crystal color with the Miller indices of the exposed sur- face. P SPIE. 2006; 6128: 61281G.
16. Simonis P, Vigneron JP. Structural color produced by a three-dimensional photonic polycrystal in the scales of a longhorn beetle: *Pseudomyagrus waterhousei* (Coleoptera: Cerambicidae). Phys Rev E 2011; 83: 011908.
17. Boher P, Leroux T, Bignon T, Blanc P. Color display evaluation vs. viewing angle using L* a* b* color space and Fourier-optics measurements. J Inf Disp. 2011; 12: 179–90.
18. Withrow RB, Withrow AP. Generation, control and measurement of visible and near-visible radiant energy. Radiat Biol. 1956; 3: 125–258.
19. Holm E, Edney EB. Daily activity of Namib desert arthropods in relation to climate. Ecology. 1973; 54: 45–56.
20. Bay A, Sarrazin M, Vigneron JP. Light extraction: What we can learn from fireflies. Proceedings SPIE. 2012; 8480: 84800G.
21. Whitehead L, Mossman M, Kushnir A. Observations of total internal reflection at a natural superhydro- phobic surface. Phys Canada. 2008; 64: 7–12.
22. Vigneron JP, Simonis P. Natural photonic crystals. Physica B. 2012; 407: 4032–4036.
23. Goldstein DH. Reflection properties of Scarabaeidae. P SPIE. 2005; 5888: 58880T.
24. Neville A, Caveney S. Scarabaeid beetle exocuticle as an optical analogue of cholesteric liquid crystals. Biol Rev. 1969; 44: 531–62. PMID: 5308457
25. Bouligand Y. Twisted fibrous arrangements in biological materials and cholesteric meso phases. Tis- sue Cell. 1972; 4: 189–217. PMID: 4600349
26. Kattawar G. A search for circular polarization in nature. P Soc Photo-Opt Ins. 1994; 5: 42–43.
27. Ghiradella HT, Butler MW. Many variations on a few themes: a broader look at development of irides- cent scales (and feathers). J Roy Soc Interface. 2009; 6: S251.
28. Simonis P, Vigneron JP, Bay A. Cylindrical Bragg mirrors on legs segments of the male Bolivian blueleg tarantula *Pamphobeteus antinous* (Araneae: Mygalomorphae: Theraphosidae). Opt Express. 2013; 21: 6979–96. doi: 10.1364/OE.21.006979 PMID: 23546081
29. Ghiradella HT. Structure and development of iridescent butterfly scales: Lattices and laminae. J Mor- phol. 1989; 202: 69–88.
30. Autumn K, Liang YA, Hsieh ST, Zesch W, Chan WP, Kenny TW, et al. Adhesive force of a single gecko foot-hair. Nature. 2000; 405: 681–5. PMID: 10864324
31. Ghiradella HT. Insect cuticular surface modifications: Scales and other structural formations. In: Casas J, Simpson SJ, editors. Advances in Insect Physiology. Burlington: Academic Press; 2010. p. 135–80.
32. Watt WB. Adaptive significance of pigment polymorphisms in *Colias* butterflies. I. Variation of melanin pigment in relation to thermoregulation. Evolution. 1968; 22: 437–58.
33. Hemmi JM, Marshall J, Pix W, Vorobyev M, Zeil J. The variable colours of the fiddler crab *Uca vomeris* and their relation to background and predation. J Exp Biol. 2006; 209: 4140–53. PMID: 17023607
34. Vigneron JP, Pasteels JM, Windsor DM, Vértesy Z, Rassart M, Seldrum T, et al. Switchable reflector in the Panamanian tortoise beetle *Charidotella egregia* (Chrysomelidae: Cassidinae). Phys Rev E. 2007; 76: 031907.

Chapter II

Proteome stability, heat hardening and heat-shock protein expression profiles in Cataglyphis desert ants

Quentin Willot, Cyril Gueydan and Serge Aron

Objective

This chapter compares patterns of expression of several candidate *hsps* genes between two species of *Cataglyphis* contrasting in their habitat: the Saharan species *C. bombycina* and the altitude species *C. mauritanica*. Our aim is to describe how, by taking into account the contribution of environment, morphology and behavior, physiological responses of heat-tolerance mechanisms can diverge between close relatives. This description integrates further with chapters III and IV, which put more emphasis on the potential molecular mechanisms underlying the divergence in heat-shock response observed between *C. bombycina* and *C. mauritanica*.

© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 1721-1728 doi:10.1242/jeb.154161

RESEARCH ARTICLE

Proteome stability, heat hardening and heat-shock protein expression profiles in *Cataglyphis* desert ants

Quentin Willot[1,*], Cyril Gueydan[2] and Serge Aron[1]

ABSTRACT

In ectotherms, high temperatures impose physical limits, impeding activity. Exposure to high heat levels causes various deleterious and lethal effects, including protein misfolding and denaturation. Thermophilic ectotherms have evolved various ways to increase macromolecular stability and cope with elevated body temperatures; these include the high constitutive expression of molecular chaperones. In this study, we investigated the effect of moderate to severe heat shock (37–45°C) on survival, heat hardening, protein damage and the expression of five heat tolerance-related genes (*hsc70-4 h1, hsc70-4 h2, hsp83, hsc70-5 and hsf1*) in two closely related *Cataglyphis* ants that occur in distinct habitats. Our results show that the highly thermophilic Sahara ant *Cataglyphis* bombycina constitutively expresses HSC70 at higher levels, but has lower induced expression of heat tolerance-related genes in response to heat shock, as compared with the more mesophilic Cataglyphis mauritanica found in the Atlas Mountains. As a result, *C. bombycina* demonstrates increased protein stability when exposed to acute heat stress but is less disposed to acquiring induced thermotolerance via heat hardening. These results provide further insight into the evolutionary plasticity of the hsp gene expression system and subsequent physiological adaptations in thermophilous desert insects to adapt to harsh environmental conditions.

KEY WORDS: Heat stress, Molecular chaperone, Heat shock response

INTRODUCTION

Protein structural stability is paramount to the maintenance of cell integrity and is highly dependent on temperature. Proteotoxic stressors, such as heat shock, can cause proteins to misfold and denature, resulting in their aggregation and a subsequent loss of biological function and an impairment of normal metabolic processes (Sørensen et al., 2003; Mayer, 2010). Accordingly, the heat-shock response (HSR) was selected for early on in evolutionary time and has been widely conserved across taxa (Moseley, 1997; Feder and Hofman, 1999). It includes the production of molecular chaperones – such as heat-shock proteins (HSPs) – that are involved in the assembly, folding and proper translocation of cellular proteins (Sørensen et al., 2003; Moseley, 1997; Feder and Hofman, 1999). Expression of HSPs depends on the activation of transcriptional heat-shock factors (HSFs). HSF1 trimerises during activation and binds to specific cis-regulatory elements in the genome – known as heat-shock elements (HSEs) – and thus induces the expression of target genes, including molecular chaperones (Sarge et al., 1993; Zatsepina et al., 2000). The constitutive expression of HSPs and their relative levels of induced expression under stressful conditions are associated with an organism's adaptive capacity to tolerate thermal pressures (Evgen'ev et al., 2014). Comparisons among species from contrasting environments have shown that thermophilic species usually have higher constitutive levels of HSPs than do their mesophilic relatives. However, their relative induced expression of HSPs is lower in response to heat stress (Rinehart et al., 2006; Dong et al., 2008; Clack and Peck, 2009; Evgen'ev et al., 2014). Moreover, in thermophiles, HSF activation and the resulting induction of HSP synthesis occur at higher temperatures. Ultimately, proteins of thermophiles are more stable than those of mesophiles, whether as a result of structural changes or as a consequence of large amounts of constitutive HSPs (Evgen'ev et al., 2014). It has been shown that both the duplication of hsp genes and shifts in HSE arrangement and position allow for greater HSR functional plasticity, helping organisms adopt the best-suited response to environmental heat stress (Bettencourt and Feder, 2001; Tian et al., 2010; Nguyen et al., 2016). The use of this system is well developed in ectotherms, which cannot regulate their body temperatures and whose metabolic processes and stability therefore depend on microclimatic conditions (Hazel, 1995; Lin and Somero, 1995; Willmer et al., 2000). Consequently, ectotherms are interesting models with which to explore HSR patterns under conditions of thermal stress (Evgen'ev et al., 2014).

Cataglyphis desert ants present a unique opportunity for investigating the HSR in heat-tolerant terrestrial ectotherms. All *Cataglyphis* species inhabit arid lands or deserts – they are found in the Sahara, the Near East, the Middle East, the Arabian Peninsula and Central Asia (Lenoir et al., 2009; Boulay et al., 2017). In contrast to many other desert-adapted species, which tend to escape the heat by being nocturnal or crepuscular, *Cataglyphis* ants take advantage of their high thermal tolerance to remain active even during the hottest parts of the day: most are scavengers and collect the bodies of less-tolerant, heat-stricken arthropods (Harkness and Wehner, 1977; Wehner et al., 1992). These ants are generally equipped with long legs, which increase the distance between their bodies and the soil; this trait reduces exposure of the body core to high ground temperatures, thus improving body cooling, and also enhances running velocity (Marsh, 1985; Sommer and Wehner, 2012). Previous studies have shown that foragers of the Saharan silver ant, *Cataglyphis* bombycina, produce what was thought to be HSP70 before emerging from the nest, thereby bypassing the need to acclimate to sudden heat exposure (Gehring and Wehner, 1995). However, more recently, it was discovered that the primary heat-induced gene hsp70 has been completely lost in Hymenoptera; instead, two *hsc70-4* paralogues are expressed (Nguyen et al., 2016). At present, the ability to express other HSPs and to induce gene expression in response to heat stress remains completely unexplored in *Cataglyphis*.

[1]Evolutionary Biology and Ecology, UniversitéLibre de Bruxelles, 1050 Brussels, Belgium. [2]Molecular Biology of the Gene, UniversitéLibre de Bruxelles, 6041 Charleroi (Gosselies), Belgium.

*Author for correspondence (Quentin.Willot@ulb.ac.be)

Q.W., 0000-0001-5955-9456

Received 29 November 2016; Accepted 17 February 2017

Journal of Experimental Biology (2017) 220, 1721-1728 doi:10.1242/jeb.154161

In this study, we compared the HSRs of two closely related *Cataglyphis* species – *C. bombycina* and *C. mauritanica* – found in natural habitats that contrast in mean annual temperature and temperature seasonality (Fig. 1). *Cataglyphis* bombycina is found in warm deserts, which are characterised by extremely high summer temperatures and warm winters. This species has one of the highest critical thermal maximum values observed among terrestrial organisms (CT_{max}=53.6°C; Hoffmann et al., 2013). Workers are active during the hottest parts of the day, when air temperatures may exceed 50°C (Wehner et al., 1992). Their lower limit for foraging activity is influenced by the presence of their main lizard predator, Acanthodactylus dumerili, which retreats to its underground burrow when temperatures becomes unbearable. Workers are thus constrained to forage within a narrow thermal window (46.5–53.6°C). *Cataglyphis* mauritanica, in contrast, lives in the Atlas Mountains, which are characterised by a cold semi-arid climate, with warm summers and cold winters. This species experiences heat stress during the summer, and their habitat exhibits extreme diurnal and seasonal temperature fluctuations that are associated with altitude (Dillon et al., 2006; Peel et al., 2007). As a result, when foraging, *C. mauritanica* workers probably face lower but much more variable temperatures than do *C. bombycina* workers.

First, we compared the species' general heat tolerance and their ability to induce thermotolerance via heat hardening (i.e. the synthesis of thermoprotective molecules following mild heat stress; Feder and Hofmann, 1999) by exposing workers to sublethal and lethal heat shocks. Second, we quantified in vivo protein damage resulting from heat shock by examining protein aggregation levels. Third, we compared the levels of constitutively expressed HSC70 between the two species. Fourth, we characterised the induced expression of several hsp genes – *hsf1*, *hsc70-4h*, *hsp83* and *hsc70-5* – known to be associated with HSR, heat tolerance and protein folding (Sarge et al., 1993; Feder and Hofmann, 1999; Nguyen et al., 2016). Based on current knowledge regarding the molecular mechanisms underlying adaptations to extreme environments (Evgen'ev et al., 2014), we predicted that the more thermophilic species, *C. bombycina*, would constitutively express molecular chaperones at higher levels than would the more mesophilic species, *C. mauritanica*. Similarly, we predicted that *C. bombycina* would show lower levels of induced hsp gene expression, reduced use of heat hardening to increase thermal tolerance, and lower levels of heat-shock-related protein damage.

MATERIALS AND METHODS

Field sampling and laboratory rearing

Colonies of *C. bombycina* Roger 1859 were collected near Zagora (30°19′56″N, 5°50′18″W), located in the Draa Valley in southern Morocco. Zagora is characterised by a warm desert climate; it experiences extremely high summer temperatures from early spring to late autumn and warm winters (mean summer temperature 33.4°C, altitude 724 m). Colonies of *C. mauritanica* (Emery 1906) were collected in Ifrane National Park (33°33′22″N, 5°14′15″W), located in the Atlas Mountains of Morocco. Ifrane is characterised by a cold semi-arid climate; it experiences warm summers and cold winters (mean summer temperature 22.7°C, altitude 1730 m; Peel et al., 2007). Colonies were kept in 30×40×10 mm plastic boxes with Fluon®-coated sides and a thin layer of clean sand on the bottom. For nesting, they were given 16×150 mm glass test tubes; the bottoms contained water, which ants could access via a moist cotton plug. The ant colonies were reared under constant environmental conditions: a temperature of 25°C, a 12 h:12 h light:dark cycle and 60% relative humidity. They were provided with a sugar solution ad libitum and sliced mealworms twice a week. They were kept under laboratory conditions for at least 2 months prior to experimentation. All the experiments were performed on workers hatched and raised in the laboratory (i.e. from the egg to adult stage).

Fig. 1. *Cataglyphis* bombycina (left) and *Cataglyphis* mauritanica (right) workers. *Cataglyphis* bombycina lives in the Sahara Desert, while *C. mauritanica* is found in the Atlas Mountains; *C. bombycina* is the more thermophilic of the two.

Thermal tolerance and heat hardening

General thermal tolerance was determined using heat-stress experiments. Three temperature treatments were used – 37, 40 and 45°C – which corresponded to mild, medium and severe heat stress. Previous studies (Gehring and Wehner, 1995) showed that HSC70 synthesis continues up to 45°C in the silver ant. For each treatment, three to six groups of 15 workers were used per species. Worker groups were placed in 50 ml glass vials containing moist cotton balls. The vials were then submerged in a digital water bath (SW22, Julabo GmbH, Seelbach, Germany); the temperature inside the vials was monitored using 0.075 mm diameter thermocouples [Type K Thermocouple (Chromel/Alumel), RS Components Ltd, Glasgow, UK] connected to a digital thermometer (RS Pro RS52 Digital Thermometer, RS Components Ltd). The ants were exposed to the treatment for 3 h. This duration of exposure was previously used to induce a significant HSR in *C. bombycina* (Gehring and Wehner, 1995). The species' use of heat hardening was assessed by pre-exposing workers to mild heat stress (37°C) for 2 h before they experienced severe heat stress (45°C) for 3 h. In both the thermal-tolerance and heat-hardening experiments, ants that could still move after the treatments were considered to be alive. We used worker survival rates as proxies for levels of thermal tolerance and heat hardening.

Protein damage

To examine the protein damage caused by heat stress, we compared protein aggregation levels between control workers, which spent 3 h at 25°C, and heat-shocked workers, which spent 3 h at 45°C; the workers were immediately flash frozen following treatment. Nine groups of six individuals were used per treatment per species. Protein aggregates were extracted and purified as per Chen et al. (2002) and Rinehart et al. (2006). Briefly, workers were pooled and placed in a chilled extraction buffer consisting of 0.1% Triton X-100, 60 mmol l^{-1} PIPES, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 100 mmol l^{-1} NaCl, 0.5 mmol l^{-1} PMSF, 0.75 mg l^{-1} leupeptin and 0.1 mmol l^{-1} DL-DTT (Sigma-Aldrich Chemie GmbH, Munich, Germany). They were then homogenised for 3 min at maximum speed in a mixer mill (MM 301, Retsch GmbH, Haan, Germany) with 2.8 mm stainless steel beads. The resulting homogenate was filtered through a 21-gauge needle (Sterican®, Braun, Kronberg, Germany) to remove large pieces of cuticle and organs. The filtrate was then centrifuged at 680 *g* for 10 min at 4°C to pellet nuclei and large cell fragments. The supernatant was centrifuged at 35,000 *g* for 14 min at 4°C (Optima™ L-70K

Journal of Experimental Biology (2017) 220, 1721-1728 doi:10.1242/jeb.154161

Ultracentrifuge, Beckman Coulter, Chaska, MN, USA) to separate the Triton-soluble fraction from the Triton-insoluble fraction (Chen et al., 2002). To purify the protein aggregates, the Triton-insoluble fraction was twice suspended, then sonicated and pelleted at 17,000 *g* for 30 min at 4°C. The resulting pellet was resuspended, sonicated and pelleted at 5000 *g* for 30 min at 4°C. Then, the pelleted protein aggregates were resuspended and assayed for protein content. Protein concentrations were determined using the Bradford procedure (BioRad, Hercules, CA, USA) and BSA as a standard (Rinehart et al., 2006).

Constitutive levels of HSC70

Constitutive levels of HSC70 were estimated by western blotting. We extracted total protein from 20 mg of randomly selected control and heat-shocked workers (see above) per species. Protein concentrations were determined using the Bradford procedure (BioRad). A 15 µg sample of the extract was mixed with 6-fold concentrated Laemmli buffer (1% SDS, 5% β-mercaptoethanol and 10% glycerin; Sigma-Aldrich Chemie GmbH) in a 1:6 ratio and kept at 95°C for 5 min. Samples were then diluted in Laemmli buffer and analysed in duplicate by SDS-PAGE on a 10% gel. The gel was stained with Coomassie Blue and the total signal for each lane was quantified using a CCD camera, which established the control loading values for each sample. Proteins were then electrotransferred onto an Amersham Protran 0.45 µm nitrocellulose blotting membrane (GE Healthcare Life Sciences, Little Chalfont, UK). Skimmed milk (1.5%) was used for blocking (1 h, 20°C) and to dilute the antibodies. The membrane was washed three times with TBS-T buffer (10 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl and 2% Tween-20) and incubated overnight at 4°C with an anti-HSP70 monoclonal mouse antibody (1/2000 dilution; clone BRM-22, Sigma-Aldrich, Rehovot, Israel). Secondary antibodies (1/10,000 dilution; anti-mouse IgG peroxidase, Sigma-Aldrich) were then added, and the solution was left at 20°C for 2 h. Signal strength was quantified using an Odyssey® FC Imaging System (Li-Cor® Bioscience, Lincoln, NE, USA).

Heat stress and gene upregulation

To examine the species' ability to induce the expression of the target hsp genes in response to heat stress, total RNA was extracted from control workers, heat-shocked workers (see above) or heat-shocked workers after 1 h respite time at 25°C. We used three groups of six randomly selected workers per treatment per species. Samples were pooled by treatment and placed in 1 ml of chilled Trizol reagent (Invitrogen, Carlsbad, CA, USA). They were then homogenised for 3 min at maximum speed in a mixer mill (MM 301, Retsch GmbH) with 2.8 mm zirconium oxide beads (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted according to the manufacturer's instructions. RNA was quantified with a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific Bvba, Gent, Belgium). The 260 nm/280 nm absorbance ratios for the samples were between 1.8 and 2.0, indicating sufficient RNA purity.

After DNase treatment (amplification-grade DNAse I, ThermoFisher Scientific, Carlsbad, CA, USA), 500 ng of total RNA was retrotranscribed using a RT-mix kit (SuperScript® III Reverse Transcriptase, ThermoFisher Scientific). Primers were designed according to quality guidelines for qPCR to detect specific target genes (Thornton and Basu, 2011; Table S1). Quantitative PCR (qPCR) was performed using an ABI StepOnePlus Real-Time PCR System (ThermoFisher Scientific). We used 20 µl reaction volumes containing 2 ng of template cDNA, 8 nmol l^{-1} of total primer and 10 µl of SYBR® (Premix Ex Taq™ II, Takara Bio USA Inc., Mountain View, CA, USA). The following temperature program was used: an initial incubation step at 95°C for 2 min, then 40 cycles at 95°C for 15 s, and finally an annealing step followed by an extension step, each at 60°C for 60 s. Melt curve analysis confirmed the presence of a single amplicon. Control qPCR reactions were performed using RNA extract as a template to confirm the absence of genomic DNA contamination. Relative changes in gene expression were calculated using the CT method (Livak and Schmittgen, 2001) and Rest software v.2.0.13 (Pfaffl et al., 2002). We screened for several commonly used housekeeping genes; the gene set we used for standardisation [i.e. *β-actin* and *eukaryotic elongation factor-1* (*eEF-1*)] was determined using both Normfinder (Andersen et al., 2004) and GeneNorm (Vandesompele et al., 2002).

Statistical analyses

Survival rates in the thermal tolerance and heat-hardening experiments, as well as levels of protein aggregation in response to heat stress, were compared using one-way analyses of variance (ANOVA). Multiple comparisons among pairs of means were carried out using Tukey *post hoc* tests. Because 100% of *C. mauritanica* workers survived in the 37 and 40°C treatments (meaning residuals did not meet the assumption of normality; see Results), we also tested for an association between survival and temperature (37, 40 and 45°C) for each species using a non-parametric Spearman's rank correlation test. Before analysing the gene expression patterns, the relative expression values were log transformed to meet assumptions of normality. The significance of treatment on gene upregulation in each species was tested using ANOVA followed by Tukey *post hoc* tests. Relative changes in gene expression between species were analysed with Student's t-tests.

RESULTS

Thermal tolerance and heat hardening

To assess general thermal tolerance, we measured the survival rates of *C. bombycina* and *C. mauritanica* workers exposed to mild (37°C), medium (40°C) and severe (45°C) heat stress (Fig. 2). *Cataglyphis* bombycina workers showed a slight, but non-

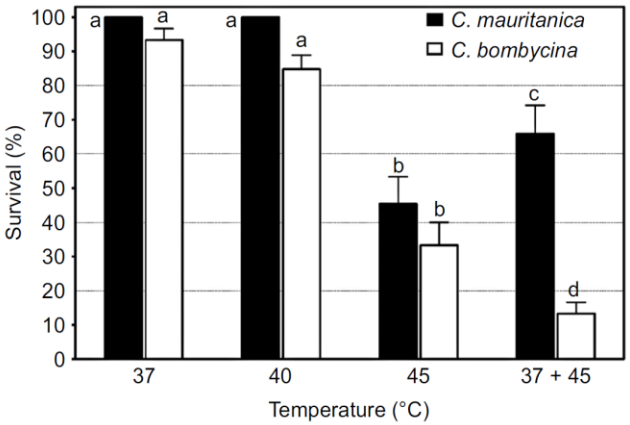

Fig. 2. Survival rate of *C. mauritanica* and *C. bombycina* workers exposed to various temperature regimes. Mean±s.d. percentage survival in three to six groups of 15 individuals each. Exposure to 37, 40 or 45°C for 3 h did not cause significant differences in mortality rates between species. Survival decreased dramatically after a 3 h, 45°C heat stress for both species. A mild heat hardening of 2 h at 37°C followed by heat stress of 3 h at 45°C significantly increased survival in *C. mauritanica* while it decreased survival in *C. bombycina*. Different lowercase letters indicate significant differences between treatments (one-way ANOVA and Tukey *post hoc* test, $P<0.05$).

Journal of Experimental Biology (2017) 220, 1721-1728 doi:10.1242/jeb.154161

significant decrease in survival rate in response to mild and medium heat stress (mean±s.d. survival rate: 93±4.4% and 88.7± 7.7%, respectively), while all the *C. mauritanica* workers in these heat-stress treatments survived (mean±s.d. survival rate: 100±0% for both temperatures). However, severe heat stress resulted in a dramatic decrease in worker survival in both species (mean±s.d. survival rate for *C. bombycina*: 33±8.9% and *C. mauritanica*: 45.5± 13.51%; one-way ANOVA: $F_{7,40}$=69.91, P<0.0001; Tukey *post hoc* test, P<0.05). Consistent with this result, Spearman's rank correlation tests showed a significant negative association between survival rate and temperature within each species (*C. bombycina*: r_s=−0.774, n=12, P=0.003; *C. mauritanica*: r_s=−0.868, n=10, P=0.002). Worker survival did not differ between species (P>0.05).

There was no evidence of heat hardening in *C. bombycina*. Rather, treatment of workers appeared to decrease survival (13.3±4.4%; P<0.05). In contrast, in *C. mauritanica*, treated workers had significantly higher survival rates (control: 45.5± 13.51% versus treated: 65.9±10.6%; P<0.05), suggesting heat hardening occurred in this species (Fig. 2).

Protein damage

We examined the protein damage resulting from heat stress by comparing protein aggregation levels between control and heat-shocked workers. Control workers had low levels of protein aggregation (mean±s.d., *C. bombycina*: 1.39±1.04 ng µg^{-1} total protein extract; *C. mauritanica*: 1.42±0.54 ng µg^{-1}; Fig. 3). When exposed to heat stress, both species showed increased levels of protein aggregation (*C. bombycina*: 5.01±2.24 ng µg^{-1}; *C. mauritanica*: 9.53±2.37 ng µg^{-1}; $F_{3,56}$=23.23, P<0.0001; Tukey *post hoc* test: P<0.05). Protein aggregation levels in heat-shocked workers were significantly higher in *C. mauritanica* than in *C. bombycina* (P<0.001).

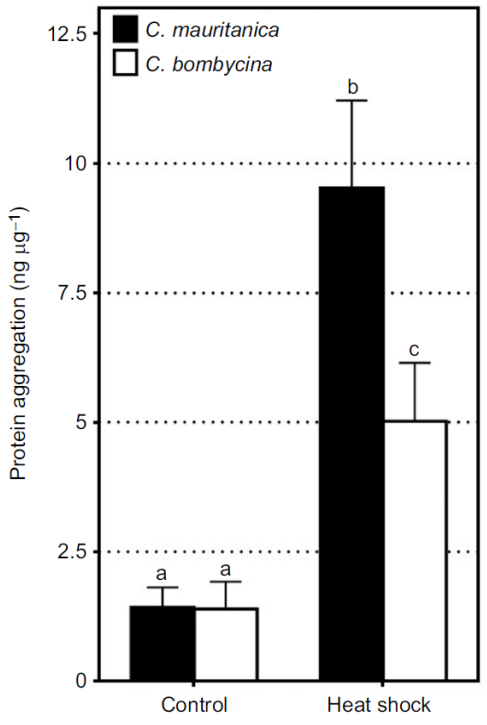

Fig. 3. Protein aggregation after heat stress in *C. mauritanica* and *C. bombycina* workers. Mean±s.d. protein aggregation (ng µg^{-1} total protein) in nine groups of six individuals each, exposed to heat shock (45°C) or control (25°C) conditions for 3 h. Both species experienced an increase in protein aggregation after heat stress; however, the increment was significantly higher in *C. mauritanica* than in *C. bombycina*. Different lowercase letters indicate a significant difference between treatments (one-way ANOVA and Tukey *post hoc* test, P<0.05).

Constitutive levels of HSC70

The concentration of HSC70 in the protein extract was assessed by western blotting and corrected for the total amount of protein loaded per sample using Coomassie Blue signal strength. The results are expressed as the ratio of HSC70 to total protein. Higher constitutive levels of HSC70 were detected in *C. bombycina* than in *C. mauritanica* (Fig. 4).

Heat stress and gene upregulation

The target hsp genes showed different expression patterns in response to heat shock. We found that hsf1 expression was not upregulated (Fig. 5A), nor did it differ between the two species. In contrast, hsp83 and hsc70-5 expression was upregulated in both species (*C. bombycina*: $F_{6,14}$=6.64, P<0.002; *C. mauritanica*: $F_{6,14}$=31.15, P<0.0001; Tukey *post hoc* tests: P<0.05 for both species; Fig. 5B,C) but no interspecific difference was found (two- tailed t-test, P>0.07 for both genes). In the case of *hsc70-4 h1*, gene expression was significantly upregulated in *C. mauritanica* (P<0.01) but not in *C. bombycina* (between-species difference: t=3.85, $d.f.$=4, P<0.02; Fig. 5D). Finally, *hsc70-4 h2* expression was upregulated in both species (P<0.01), and the species differed in their expression patterns (t=3.67, $d.f.$=4, P<0.03; Fig. 5E).

DISCUSSION

Compared with related mesophilic species, thermophilic species are expected to have higher constitutive expression but lower induced expression of HSP-related genes (Evgen'ev et al., 2014). The results of our study support this prediction: in the highly thermophilic *C. bombycina*, constitutive expression of HSC70 was higher than in *C. mauritanica*, but induced expression of *hsc70-4 h1* and *hsc70-4*

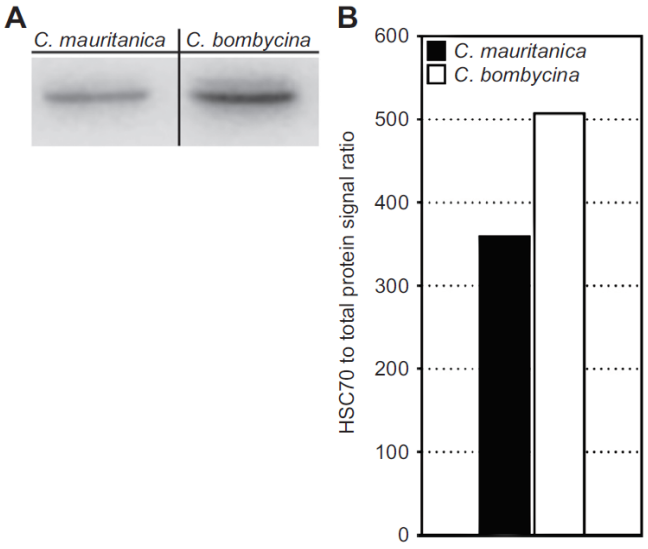

Fig. 4. Western blotting of HSC70 proteins from *C. mauritanica* and *C. bombycina* workers. Total protein extracts were separated and the presence of HSC70 was assessed using monoclonal antibodies. (A) Moderate levels of HSC70 were detected in *C. mauritanica* while higher levels of HSC70 were detected in *C. bombycina*. (B) HSC70 normalised to total protein signal ratio. Results are representative of three replicates.

Journal of Experimental Biology (2017) 220, 1721-1728 doi:10.1242/jeb.154161

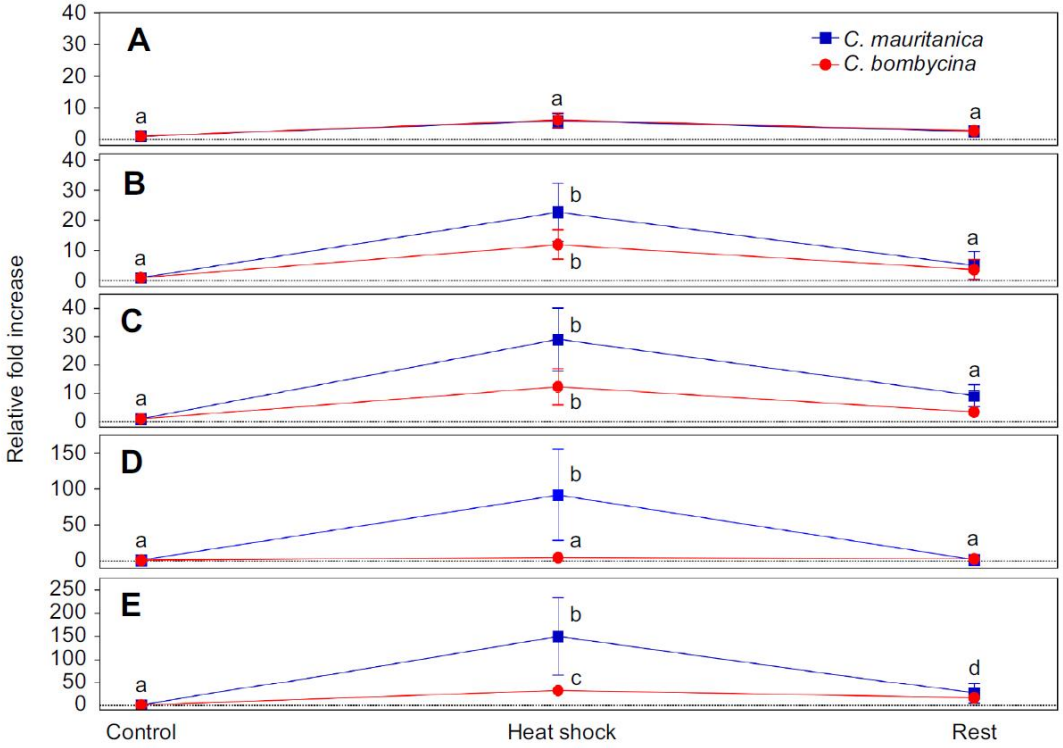

Fig. 5. Relative fold increase in expression of six genes in response to a heat shock in *C. mauritanica* and *C. bombycina* across different treatments. Total RNA was extracted from three groups of six individuals each in control conditions (25°C for 3 h), exposed to heat shock (40°C for 3 h) or subsequently rested (25°C for 1 h). Expression of (A) *hsf1*, (B) *hsc70-5*, (C) *hsp83*, (D) *hsc70-4 h1* and (E) *hsc70-4 h2* was normalised to that of *β-actin* and *eEf-1* (see Materials and methods). Mean±s.d. fold change is presented. Different lowercase letters indicate a significant difference between treatments (one-way ANOVA and Tukey *post hoc* test, $P<0.05$).

h2 in response to heat shock was lower. Furthermore, *C. bombycina* workers suffered less protein damage upon heat shock and exhibited lower rates of heat hardening. Although the strength of inference that can be drawn from comparison of two species may be limited (Garland and Adolph, 1994), our study strongly supports that the HSR is highly correlated to the species' thermal environment.

Heat stress and gene upregulation

Few studies have investigated HSP synthesis under heat-stress conditions in ants. For those that have, HSP70 synthesis has largely been the focus (Gehring and Wehner, 1995; Maisov et al., 2007). However, Nguyen et al. (2016) recently showed that the primary inducible gene involved in HSR in Drosophila – *hsp70* – has been completely lost in Hymenoptera, which instead employs two *hsc70-4* paralogues. Like Gehring and Wehner (1995), we found that HSC70 is constitutively expressed in *C. bombycina*. We also found comparatively moderate levels of HSC70 in *C. mauritanica* under control conditions. High constitutive expression of molecular chaperones has been reported in a number of ectotherms living under stressful conditions (e.g. elevated temperature, high salinity), including molluscs (Dong et al., 2008), echinoderms (González et al., 2016), dipterans (Rinehart et al., 2006; Garbuz et al., 2008) and lizards (Zatsepina et al., 2000). The sustained production of HSC70 by *C. bombycina* and *C. mauritanica* is probably an adaptation for coping with heat. We expect elevated constitutive expression of HSC70 to occur across the entire *Cataglyphis* genus, as all its members are thermophilic.

To the best of our knowledge, only three studies have explored the expression of HSP-related genes in response to heat stress in ants. Beside the elevated constitutive level of HSC70 in *C. bombycina*, Gehring and Wehner (1995) also showed that HSC70 synthesis carries on at temperatures as high as 45°C in this species. In contrast, this synthesis is abolished above 40°C in the wood ant Formica polyctena, a mesic species closely related to *Cataglyphis* that inhabits the northern part of the Paleartic region. The second study investigated *hsp60*, *hsp75* and *hsp90* expression in response to temperature in the wood ant Formica cinerea and found no significant effects (Slipinski et al., 2015). The third study compared heat-shock-related expression patterns of several hsp genes between Pogonomyrmex barbatus, an ant living in hot and arid climates, and Aphaenogaster picea, an ant living in cool woodlands (Nguyen et al., 2016). These authors showed that expression of *hsc70-4*, *hsp83* and *hsp40* was upregulated in response to heat stress and the species differed significantly in relative expression levels. More specifically, upregulation occurred at higher temperatures in P. barbatus. In contrast, induced expression of *hsc70-5*, *hsc70-3* and *hsp60* was unaffected by heat stress. Our findings add to the knowledge about HSR in ants. They show that, in *Cataglyphis*, hsf1 expression is not induced by heat shock. This result suggests that ambient concentrations of cytosolic HSF1 are sufficient to induce the expression of hsp genes under conditions of heat stress. Consistent with this hypothesis, in previous studies, differences in HSF1 activation and binding to HSEs, as opposed to upregulation, are crucial to the constitutive and induced expression of HSPs (Zatsepina et al., 2000). In response to heat shock, *hsp83* expression was moderately upregulated in both *C. bombycina* and *C. mauritanica*. Upregulation of the *hsp90* gene family in response to heat stress is well documented in ectotherms (Rinehart et al., 2007; Choi et al., 2014; González et al., 2016; Nguyen et al., 2016) and, based on our findings, also appears to be conserved in *Cataglyphis*. We also observed an increase in *hsc70-5* expression in response to heat shock. This result was somewhat unexpected as this cognate has

comparison of HSC70 constitutive expression in *C. bombycina* might reveal key differences in heat tolerance strategies.

Finally, in all our experiments the ants were exposed to a 3–5 h heat stress. This corresponds to somewhat artificial conditions, as *C. bombycina* foragers frequently use thermal refuges in order to dump excessive internal heat in the wild (Wehner et al., 1992). The length of exposure to heat stresses in our experiments was chosen to take into account the full extent of the HSR and compare results with existing data (Gehring and Wehner, 1995). We showed that in both species of *Cataglyphis* studied, mRNA of up-regulated genes following heat shock abruptly decreased after 1 h respite time (Fig. 5B–E). We are therefore confident that the lessened *hsc70* mRNA induction observed in *C. bombycina* does not result from an experiment in which 4 h at 40°C would allow significant mRNA decay.

Conclusions

In summary, this study demonstrates that the more thermophilic ant species, *C. bombycina*, has higher constitutive expression of HSC70 and lower induced expression of *hsp* genes in response to heat stress than the more mesophilic ant species *C. mauritanica*. Consequently, *C. bombycina* workers show increased protein stability when exposed to heat stress but are less prone to acquiring induced thermotolerance by means of heat hardening. These results confirm that HSR patterns are highly representative of the species' thermal environment. More generally, our findings provide insights into the physiological plasticity needed to cope with fluctuating or novel temperature regimes. For example, global warming might prove to be an even greater challenge for species with less-plastic HSP systems than for species that are better equipped to cope with fluctuating body temperatures.

Acknowledgements

We thank E. Goormaghtigh and G. Vandenbusshe for their advice concerning the protein aggregation experiments and the use of their laboratory. We are grateful to R. Soin and A. Nazih for their help with the RTq-PCR analyses and the western blots. Thanks to J. Pearce for her language editing services.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Q.W., C.G. and S.A. conceived and planned the study. Q.W. and S.A. collected samples. Q.W. and C.G. performed molecular work and analysed the data. All authors contributed to drafting the article, approved the final published version and agree to be held accountable for all aspects of the work.

Funding

This work was supported by a Fonds pour la Formation àla Recherche dans l'Industrie et dans l'Agriculture (FRIA) scholarship (to Q.W.) and CDR funding (to S.A.; J.0151.16) from the Belgian Fund for Scientific Research (Fonds de la Recherche Scientifique, FRS-FNRS).

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.154161.supplemental

References

Andersen, C. L., Jensen, J. L. and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245-5250.

Bahrndorff, S., Mariën, J., Loeschcke, V. and Ellers, J. (2009). Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail, *Orchesella cincta*. *Funct. Ecol.* **23**, 233-239.

Bettencourt, B. R. and Feder, M. E. (2001). Hsp70 duplication in the *Drosophila melanogaster* species group: how and when did two become five? *Mol. Biol. Evol.* **18,** 1272-1282.

Boulay, R., Aron, S., Cerdá, X., Doums, C., Graham, P., Hefetz, A. and Monnin, T. (2017). Social life in arid environments: the case study of *Cataglyphis* ants. *Annu. Rev. Entomol.* **62**, 305-321.

Chen, Q., Ma, E., Behar, K. L., Xu, T. and Haddad, G. G. (2002). Role of trehalose phosphate synthase in anoxia tolerance and development in *Drosophila melanogaster*. *J. Biol. Chem.* **277**, 3274-3279.

Choi, B.-G., Hepat, R. and Kim, Y. (2014). RNA interference of a heat shock protein, Hsp70, loses its protection role in indirect chilling injury to the beet armyworm, *Spodopteraexigua*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **168**, 90-95.

Clark, M. S. and Peck, L. S. (2009). HSP70 heat shock proteins and environmental stress in Antarctic marine organisms: a mini-review. *Mar. Genomics* **2**, 11-18.

Dillon, M. E., Frazier, M. R. and Dudley, R. (2006). Into thin air: physiology and evolution of alpine insects. *Integr. Comp. Biol.* **46**, 49-61.

Dong, Y., Miller, L. P., Sanders, J. G. and Somero, G. N. (2008). Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress. *Biol. Bull.* **215**, 173-181.

Evgen'ev, M. B., Garbuz, D. G. and Zatsepina, O. G. (2014). *Heat Shock Proteins And Whole Body Adaptation To Extreme Environments*. Berlin-New-York-London: Springer.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 61, 243-282.

Garbuz, D. G., Zatsepina, O. G., Przhiboro, A. A., Yushenova, I., Guzhova, I. V. and Evgen'ev, M. B. (2008). Larvae of related Diptera species from thermally contrasting habitats exhibit continuous up-regulation of heat shock proteins and high thermotolerance. *Mol. Ecol.* **17**, 4763-4777.

Garland, T., Jr and Adolph, S. C. (1994). Why not to do two species comparative studies: limitations on inferring adaptation. *Physiol. Biochem. Zool.* **67**, 797-828.

Gehring, W. J. and Wehner, R. (1995). Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara Desert. *Proc. Natl. Acad. Sci. USA* **92**, 2994-2998.

González, K., Gaitán-Espitia, J., Font, A., Cárdenas, C. A. and González-Aravena, M. (2016). Expression pattern of heat shock proteins during acute thermal stress in the Antarctic sea urchin, *Sterechinus neumayeri*. *Rev. Chil. Hist. Nat.* **89**, 1.

Harkness, R. D. and Wehner, R. (1977). *Cataglyphis*. *Endeavour* 1, 115-121.
Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42.

Hoffmann, A. A., Chown, S. L. and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they? *Funct. Ecol.* **27**, 934-949.

Lenoir, A., Aron, S., Cerda, X. and Hefetz, A. (2009). *Cataglyphis* desert ants: a good model for evolutionary biology in Darwin's anniversary year? *A review. Isr. J. Zool.* **39**, 1-32.

Lin, J. J. and Somero, G. N. (1995). Temperature-dependent changes in expression of thermostable and thermolabile isozymes of cytosolic malate dehydrogenase in the eurythermal goby fish *Gillichthys mirabilis*. *Physiol. Zool.* **68**, 114-128.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402-408.

Maisov, A. V., Podlipaeva, Y. I. and Kipyatkov, V. E. (2007). Expression of stress proteins of HSP70 family in response to cold in *Myrmica* ants from various geographic populations. *Cell Tissue Biol.* **1**, 434-438.

Marsh, A. C. (1985). Microclimatic factors influencing foraging patterns and success of the thermophilic desert ant, *Ocymyrmex barbiger*. *Insectes Soc.* **32**, 286-296.

Mayer, M. P. (2010). Gymnastics of molecular chaperones. *Mol. Cell.* **39**, 321-331.

Moseley, P. L. (1997). Heat shock proteins and heat adaptation of the whole organism. *J. Appl. Physiol.* **83**, 1413-1417.

Nguyen, A. D., Gotelli, N. J. and Cahan, S. H. (2016). The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC Evol. Biol.* **16**, 15.

Peel, M. C., Finlayson, B. L. and McMahon, T. A. (2007). Updated world map of the Köppen-Geiger climate classification. *Hydrol. Earth Syst. Sci. Discuss.* **4**, 439-473.

Pfaffl, M. W., Horgan, G. W. and Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36-e36.

Rinehart, J. P., Hayward, S. A. L., Elnitsky, M. A., Sandro, L. H., Lee, R. E. and Denlinger, D. L. (2006). Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proc. Natl. Acad. Sci. USA* **103**, 14223-14227.

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. and Denlinger, D. L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proc. Natl. Acad. Sci. USA* **104**, 11130-11137.

Sarge, K. D., Murphy, S. P. and Morimoto, R. I. (1993). Activation of heat shock

gene transcription by heat shock factor I involves oligomerization, acquisition of DNA-binding activity and nuclearlocalization and can occur in the absence of stress. *Mol. Cell. Biol.* **13**, 1392-1407.

Shi, N. N., Tsai, C.-C., Camino, F., Bernard, G. D., Yu, N. and Wehner, R. (2015). Keeping cool: Enhanced optical reflection and radiative heat dissipation in Saharan silver ants. *Science* **349**, 298-301.

Ślipiński, P., Pomorski, J. J. and Kowalewska, K. (2015). Heat shock proteins expression during thermal risk exposure in the temperate xerothermic ant *Formica cinerea*. *Sociobiology* **62**, 457-459.

Sommer, S. and Wehner, R. (2012). Leg allometry in ants: extreme long-leggedness in thermophilic species. *Arthropod Struct. Dev.* **41**, 71-77.

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025-1037.

Thornton, B. and Basu, C. (2011). Real-time PCR (qPCR) primer design using free online software. *Biochem. Mol. Biol. Educ.* **39**, 145-154.

Tian, S., Haney, R. A. and Feder, M. E. (2010). Phylogeny disambiguates the evolution of heat-shock cis-regulatory elements in *Drosophila*. *PLoS ONE* **5**, e10669.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, 1.

Wehner, R., Marsh, A. C. and Wehner, S. (1992). Desert ants on a thermal tightrope. *Nature* **357**, 586-587.

Willmer, P., Stone, G. and Johnston, I. (2000). The nature and levels of adaptation. In *Environmental Physiology of Animals* (ed. P., Willmer G., Stone and I., Johnston), pp. 1-17. USA : Blackwell.

Willot, Q., Simonis, P., Vigneron, J.-P. and Aron, S. (2016). Total internal reflection accounts for the bright color of the Saharan silver ant. *PLoS ONE* **11**, e0152325.

Yocum, G. D. and Denlinger, D. L. (1993). Induction and decay of thermosensitivity in the flesh fly, *Sarcophaga crassipalpis*. *J. Comp. Physiol. B* **163**, 113-117.

Zatsepina, O. G., Ulmasov, K. A., Beresten, S. F., Molodtsov, V. B., Rybtsov, S. A. and Evgen'ev, M. B. (2000). Thermotolerant desert lizards characteristically differ in terms of heat-shock system regulation. *J. Exp. Biol.* **203**,1017-1025.

Zatsepina, O. G., Przhiboro, A. A., Yushenova, I. A., Shilova, V., Zelentsova, E. S., Shostak, N. G., Evgen'ev, M. B. and Garbuz, D. G. (2016). A *Drosophila* heat shock response represents an exception rather than a rule amongst Diptera species. *Insect. Mol. Biol.* **25**, 431-449.

Journal of Experimental Biology 220:
doi:10.1242/jeb.154161: Supplementary information

Table S1. Primer sets for qPCR including housekeeping genes.

Gene	Primer 5'-3'	Amplicon Length (bps)
β-actin (forward)	TATTATTGCTACACCTTTCCCTAAG	
β-actin (reverse)	TTCGACTAACAGATCCAACTAAAC	76
Ef1-β (forward)	AGAAAGCCCAGAAGAAGAAATAAC	
Ef1-β (reverse)	CACCCATATTCGCACGGA	76
hsc70-4_1 (forward)	CTCCGCTCTCTCTGGGTATC	
hsc70-4_1 (reverse)	CGGGATGGTAGTGTTCCTCTT	74
hsc70-4_2 (forward)	ACCGACTAGAACTATACTGTGAAC	
hsc70-4_2 (reverse)	TTGAACCCGTCGAGAAAGC	74
hsc70-5 (forward)	ACATGGTTGAATAGTACGCTTAA	
hsc70-5 (reverse)	TGAATCTTAAACTGTCCAGAAGTAA	74
hsp83 (forward)	TGACGAAATGTGCTCTCTTAAAG	
hsp83 (reverse)	AGTGATGTAGTAAATGTGCTTCTG	73
hsf1 (forward)	TGTAGTATGTATTGTAACTCTCAGTGA	
hsf1 (reverse)	CTCCATTCTCCAACATCCTAGATT	80

Chapter III

Molecular chaperoning helps safeguarding mitochondrial integrity and motor functions in the Sahara silver ant *Cataglyphis bombycina*

Quentin Willot, Patrick Mardulyn, Matthieu Defrance, Cyril Gueydan & Serge Aron

---

Objective

In the previous chapter, we show that heat-shock response regulation and mechanisms can diverge even between close relative as a response their respective habitat. Here, we explore a larger panel of those mechanisms underlying heat-tolerance using RNA-sequencing, in *C. bombycina*. Because the comparative framework outside heat-shock proteins is too fragmental in ants (as well as other non-model organisms) to provide relevant ground for comparisons, our data were interpreted mostly in the light of Hsps expression and their potential mechanisms of actions. We speculate on physiological outcomes of their up-regulation and their relation with the silver ant ecology. This chapter integrates further with chapter IV, in a sense that the same comparative approach has been yielded in another thermal scavenging species of ants. This will allow, in time, an extensive comparison of mechanisms allowing thermal scavenging ants to tolerate transient bursts of intense heat-stresses.

SCIENTIFIC REPORTS

OPEN

Molecular chaperoning helps safeguarding mitochondrial integrity and motor functions in the Sahara silver ant *Cataglyphis bombycina*

Received: 21 November 2017
Accepted: 6 June 2018
Published online: 15 June 2018

Quentin Willot[1], Patrick Mardulyn[1], Matthieu Defrance[2], Cyril Gueydan[3] & Serge Aron[1]

The Sahara silver ant *Cataglyphis bombycina* is one of the world's most thermotolerant animals. Workers forage for heat-stricken arthropods during the hottest part of the day, when temperatures exceed 50 °C. However, the physiological adaptations needed to cope with such harsh conditions remain poorly studied in this desert species. Using transcriptomics, we screened for the most heat-responsive transcripts of *C. bombycina* with aim to better characterize the molecular mechanisms involved with macromolecular stability and cell survival to heat-stress. We identified 67 strongly and consistently expressed transcripts, and we show evidences of both evolutionary selection and specific heat-induction of mitochondrial-related molecular chaperones that have not been documented in *Formicidae* so far. This indicates clear focus of the silver ant's heat-shock response in preserving mitochondrial integrity and energy production. The joined induction of small heat-shock proteins likely depicts the higher requirement of this insect for proper motor function in response to extreme burst of heat-stresses. We discuss how those physiological adaptations may effectively help workers resist and survive the scorching heat and burning ground of the midday Sahara Desert.

Temperature plays a key role in protein homeostasis[1]. Most peptides are stable within a narrow thermal range, and increases or decreases in temperature can cause them to unfold and form denatured aggregates[1,2]. Such sensitivity likely led to the early evolutionary appearance of the heat-shock response (HSR)[3,4]. One of the HSR's main functions is to increase macromolecular stability, which helps organisms cope efficiently with thermal shifts, as well as oxidative stress, heavy metal contamination, or exposure to toxins[5,6]. A major component of the HSR is the transcriptional response, which is controlled by several factors including the evolutionarily conserved transcriptional activator heat-shock factor 1 (HSF1)[7]. HSF1 trimerizes upon heat shock and binds to consensus heat-shock elements (HSEs) localized on promotor regions of target genes[8]. This response, triggered by the presence of unfolded proteins, leads to the fast and transient transcription of target genes, such as heat-shock proteins (*hsps*). Hsps are a large family of molecular chaperones. Their upregulation and accumulation are associated with thermal hardiness[9,10]. Therefore, the HSR in general, and especially Hsps production, play a central role in allowing cells to survive deleterious conditions.

Using transcriptomics, we examined the predominant molecular level processes involved with macromolecular stability and cell survival in the Sahara silver ant, *Cataglyphis bombycina*, focusing on heat-shock proteins. This species forages during the hottest part of the day, scavenging the bodies of less tolerant, heat-stricken arthropods[11]. Workers thus experience harsh conditions: air and ground temperatures can reach as high as 50 °C and 70 °C, respectively[11]. The silver ant manages this feat thanks to its remarkable ability to survive elevated body temperatures (CT_{max} = 53.6 °C)[12]. Previous studies have shown that foragers exhibit high constitutive levels of

[1]Evolutionary Biology and Ecology, Université Libre de Bruxelles, CP 160/12, Av. F.D. Roosevelt, 50, Brussels, 1050, Belgium. [2]Interuniversity Institute of Bioinformatics in Brussels, Université Libre de Bruxelles, Boulevard du Triomphe, Brussels, 1050, Belgium. [3]Molecular Biology of the Gene, Université Libre de Bruxelles, Rue des Profs. Jeener et Brachet, 12, Gosselies, 6041, Belgium. Correspondence and requests for materials should be addressed to Q.W. (email: Quentin.Willot@ulb.ac.be)

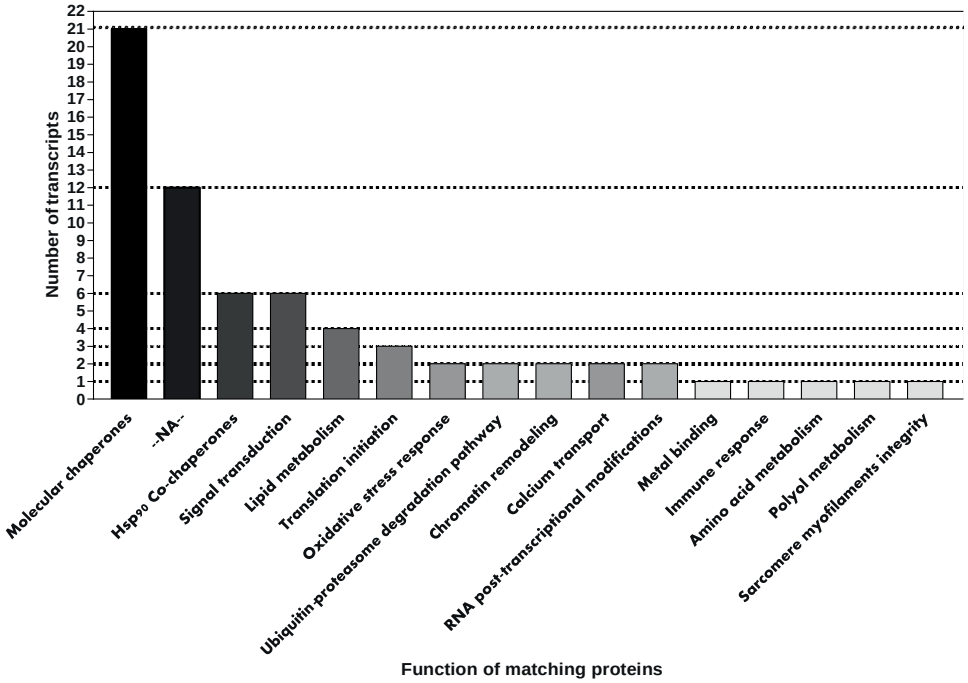

Figure 1. Functional classification of the 67 transcripts with strong and consistent heat-induced expression. Twenty-one transcripts matched up with heat-shock proteins and molecular co-chaperones (31%). Smaller groups of transcripts (<10% each) matched up with proteins with roles ranging from cell-signal transduction to sarcomere myofilament organization.

heat-shock cognate 70 (Hsc70)[13,14], suggesting that the ants can handle sudden heat exposure without needing to acclimate. However, a deeper understanding of mechanisms involved with the ability of cells to survive heat stress while maintaining high metabolic requirements associated with foraging is still lacking in the silver ant. Our aim in this study was to gain a better understanding of the molecular response underlying *C. bombycina*'s ability to survive such elevated body temperatures for short periods of time.

Results

Identification of heat-induced transcripts. We performed a differential gene expression analysis between 4 groups of heat-stressed (4 h; 45 °C) and 4 groups of control workers (25 °C, 4 h). A total of 301,363 puta- tive transcripts (including isoforms) were identified. After removing transcripts with low expression levels, 40,988 transcripts remained. Of these, 533 displayed a significant regulation in response to heat stress (FDR < 0.05; Fig. S1) and were qualified as differentially expressed sequences (DESs). Expression was downregulated for 147 DESs and upregulated for 386 DESs. Most displayed a high degree of fold change (FC) between the two condi- tions; there was also marked variance in transcription within the heat-stress treatment.

Similarity annotation. When the 533 DESs were queried against the NCBI nr protein database, 466 sequences (87%) with a high degree of homology were retrieved. Annotation was reliable, as most hits had e-values of less than $1e^{-180}$ (Fig. S2).

To better characterize the number and function of the genes involved in the HSR, we further filtered the tran- scripts. A given transcript was retained only if (*i*) mean FC was greater than 2 between heat-stressed and control ants; (*ii*) FDR was less than 0.05; and (*iii*) the relative standard deviation (RSD) of expression between biological replicates was less than 0.4. We were thus left with a total of 67 strongly and consistently expressed transcripts. For this transcript subset, the most represented taxa for the best hit of each match were mainly other ant species, particularly those in the subfamily *Formicinae*, such as *Camponotus floridanus*, a result that reflects *C. bombyci- na*'s phylogenetic history (Fig. S3).

Gene ontology annotation. Based on sequence homology, GO terms could be assigned to 393 (73%) of the 533 DESs. The transcripts were distributed across the three GO-classification domains: cellular component (GO levels 5–8), biological process (GO levels 4–8), and molecular function (GO levels 3–5) (Fig. S4).

Fifty-five of the 67 transcripts matched up with various types of proteins, most commonly Hsps and molecular co-chaperones (21 transcripts; 32%; Fig. 1). Notably, smaller groups of transcripts (less than 10% each) matched up with proteins with roles ranging from HSP90 co-chaperones involved in cell division to chromatin remod- elling and sarcomere myofilament organization. Results, including the FC in expression, are depicted in Fig. 2. Complete heat-map of heat-induced transcripts is displayed in Fig. S5.

Molecular chaperones involved in the heat-shock response. Of the 533 DESs, 36 were associ- ated with either molecular chaperones or with co-chaperones involved in protein folding. Of the 67 strongly

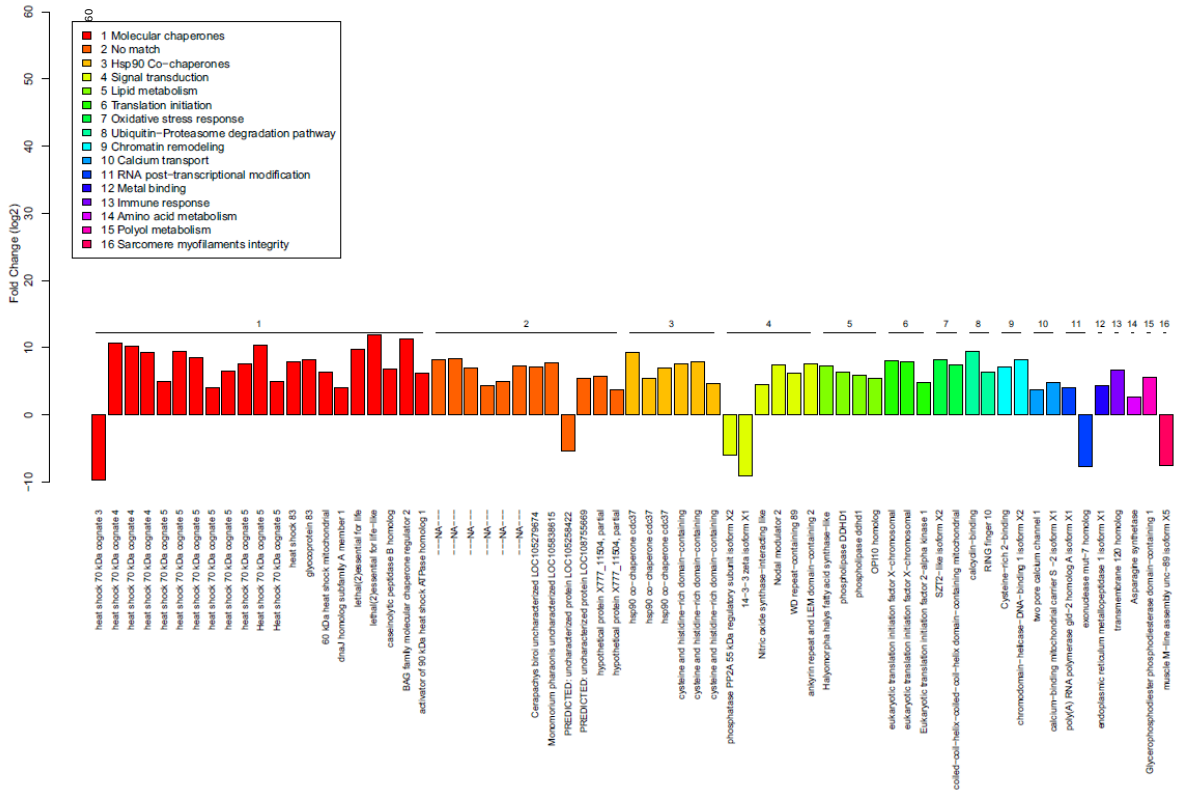

Figure 2. Average fold changes of the 67 strongly and consistently expressed transcripts induced by heat-stress in *C. bombycina* workers. The numeric colour-codes correspond to the following functional classes of proteins: 1: Molecular Chaperones; 2: No match; 3: Hsp90 co-chaperones; 4: Cell signal transduction proteins; 5: Lipid metabolism enzymes; 6: Translation initiation factors; 7: Oxidative stress response proteins; 8: Proteins involved with the ubiquitin-proteasome degradation pathway; 9:Chromatin remodeling proteins; 10: Calcium transport proteins; 11: RNA-modifying proteins; 12: Metal binding proteins; 13: Proteins involved in the immune response; 14: Proteins involved in the amino-acid metabolism; 15: proteins involved in the polyol metabolism; 16: proteins involved in the sarcomere organization.

and consistently expressed transcripts, 21 (31%) of such transcripts remained, indicating that chaperones and co-chaperones were a major part of the HSR (Fig. 1). We found members among the 5 major conserved families of Hsps (Table 1): one transcript matched up with the caseinolytic peptidase B homolog protein (ClpB) which belongs to the Hsp100 family, two with proteins in the Hsp90 family (namely the Gp93 protein and Hsp83), twelve with Hsp70 proteins, one with the Hsp60 mitochondrial molecular chaperone and two with the protein protein Efl21l encoded by *lethal(2)essential for life (l(2)efl)*, which is a member of the Hsp20 family. Among molecular co-chaperones, one transcript matched up with DnaJ homolog subfamily A member 1 (DnajA1) which belongs to the Hsp40 family, one with the BCL-2-associated athanogene protein 2 (BAG-2), and one transcript was asso- ciated with the activator of 90-kDa heat-shock protein ATPase homolog 1 (AHA1).

Under conditions of heat stress, it appeared that the transcripts associated with Hsc70-4, protein Efl21l, and BAG-2 were the most upregulated (>10 FC). The only transcript strongly downregulated by heat stress (>8 FC) was associated with Hsc70-3 (Fig. 2).

KEGG annotation of the transcripts. For the 533 DESs, the main KEGG pathways involved ribosomes (71; 13%), metabolic processes (54; 10%), secondary metabolite biosynthesis (22; 4%), protein processing in the endoplasmic reticulum (ER) (18; 3%) (Fig. S6), and PI3K-Akt signaling (14; 3%) (Fig. S7) which is part of cell cycle regulation and apoptosis. For the transcripts associated with metabolic processes, there was clear enrich- ment in the non-oxidative pentose phosphate pathway and the lipid metabolic pathway.

For the 67 strongly and consistently expressed transcripts, the two top KEGG pathways were protein process- ing in the ER (8; 11%) and PI3K-Akt signaling (4; 6%).

Detection of patterns of selection. All d_N/d_S ratios calculated for 32 coding sequences (CDS) between *C. bombycina* and the closely related ant *Camponotus floridanus* were largely below 1, with values ranging from 0.01 to 0.29 (Tables S1 and S2). This shows that all analyzed CDS are predominantly under negative selection, which is typical for a functional protein coding gene since non-synonymous mutations are more likely to generate a disadvantageous allele than an advantageous one.

However, even for proteins characterized by a d_N/d_S ratio below 1, a signal of positive selection can be detected by highlighting a specific lineage for which this ratio is significantly higher than the background ratio estimated for

Protein family	Protein	Number of associated transcripts	Significant selection along the *Cataglyphis* lineage	*Cataglyphis*-specific heat induction
Molecular chaperones				
Hsp100	ClpB	1	−	N.A
Hsp90	Hsp83	1	−	−
	Gp93	1	−	N.A
Hsp70	Hsc70-5	8	+	+
	Hsc70-4h1	2	−	−
	Hsc70-4h2	1	+	−
	Hsc70-3	1	−	N.A
Hsp60	Hsp60 mitochondrial	1	−	+
Hsp20	Efl21	2	−	+
Co-chaperones				
Hsp40	DnajA1	1	−	−
BAG proteins	BAG2	1	−	N.A
AHA1	AHA1	1	−	N.A

Table 1. Summary of the molecular chaperones and folding co-chaperones found among the 67 consistently heat-induced transcripts of the ant *C. bombycina*, queried against the NCBI non-redundant protein database (arthropod records only) using BLASTX ($<10e^{-5}$), complemented by their detected significant selection along the *Cataglyphis* lineage, and their detected specific heat induction as compared to other ants (N.A: not applicable; heat-induction of the gene has not been tested in other ant genera).

all other lineages. From the four transcripts for which we have conducted this test (Table S2), a signal of positive selection was detected for the CDS sequences of *hsc70-4 h2* (d_N/d_S ratio 3.8 times larger along the branch leading to *Cataglyphis*; *p*-value < 0.01) and of *hsc70-5* (d_N/d_S ratio 2.3 times larger along the branch leading to *Cataglyphis* and along the branches inside the clade *Cataglyphis*; *p*-value < 0.05; Table 1). These results provide some evidence that positive selection occurred more frequently for these two genes in *Cataglyphis*, compared to what happened in other ant species, *i.e.* that a higher proportion of non-synonymous mutations were favored by selection.

Discussion

So far, understanding the molecular level processes related to heat tolerance in eusocial Hymenoptera (ants, bees, wasps) has been limited to phylogeny and induction patterns of some Hsps across species and genera[13–17]. Our study investigates further gene expression patterns in response to heat stress using DGE analysis. It shows that of the 67 strongly and consistently expressed transcripts, 21 were linked to proteins that exercise either direct or indirect molecular chaperone folding activity (31%). This protein class was therefore the most responsive to heat stress. As compared, only two transcripts matched up with proteins in the ubiquitin-proteasome path- way (RNF10; a member of the E3 ubiquitin ligase family and CACYBP; which may regulate calcium-dependent ubiquitination and degradation of target peptides)[18]. Higher eukaryotes tend to rely more on refolding to clear misfolded proteins while bacteria tend to exploit degradation pathways[19,20]. Accordingly, our results suggest that

C. bombycina invests more in its protein-refolding machinery in response to heat stress than in maintaining proteostasis by increased turnover of damaged peptides.

Molecular chaperones are essential for protein synthesis, folding, and translocation under both normal and stressful conditions[9]. Among them, the five major conserved families of Hsps were represented (in order of prom- inence): Hsp70s, Hsp90s, Hsp60s, Hsp100s and small Hsps (Table 1). Here, we document heat-inducibility for the first time among ants for several of them: *hsc70-5*, *hsp-60 mitochondrial*, as well as the small heat-shock protein *l(2)efl*. Analysis of *C. bombycina* molecular chaperones associated transcripts showed that the Hsp70 family was the most prominent—it was associated with 12 transcripts. Among this family, *hsc70-4* and *hsc70-3* were the only two transcripts already expressed at 25 °C. While similar to other ants, *hsc70-4* exhibited among the greatest induction in expression[14–16], *hsc70-3* was the only molecular chaperone to show down-regulation in response to heat-stress. The effects of such down-regulation remain so far unclear with regards to molecular chaperoning and stress-tolerance and would deserve further investigation.

We performed tests of positive selection on the coding sequences belonging to the 67 consistently and strongly expressed transcripts in response to heat stress from multiple ant species (see Tables S1 and S2). They indicated a significant increase of positive selection for *hsc70-4 h2* and *hsc70-5* in the *Cataglyphis* lineage. All *Cataglyphis* species are thermal scavengers known to forage at the warmest hours of the day[21]. Consequently, specific evolu- tion of (at least some) molecular chaperones likely occurred in the genus to provide stronger support for mac- romolecular stability. Remarkably, we found eight isoforms of *hsc70-5*. This finding makes *hsc70-5* isoforms the most numerous *hsps* whose expression is upregulated in response to heat stress in this species. Previous qPCR experiments confirmed heat-inducible expression of *hsc70-5* in *Cataglyphis*[14]. In contrast, the gene *hsc70-5* was not shown to be heat-inducible in the wood ant *Aphaenogaster picea* nor in the harvester ant *Pogonomyrmex bar- batus*[15]. Canonical forms of HSEs in the promoter region of *hsc70-5* are variable and lacking in many ants[15], which could partly explain the observed divergences in induction patterns among genera. Such confirmed, evolutionary

selected and highly diversified use of *hsc70-5* in response to heat-stress seems thus so far to be restricted, among ants, to *Cataglyphis*. Importantly, it has been documented that *hsc70-5* plays a critical role in maintaining mito- chondria morphology and cellular homeostasis: knockdown of *hsc70-5* in *Drosophila melanogaster* results in severe mitochondria dysfunction as well as reduced viability, locomotion impairment, body posture defects, and reduced ATP levels[22,23]. Consistently, our results in the silver ant show a significant heat-inducibility of *hsp60* (coding for the 60 kDa heat shock protein mitochondrial) that was not reported in other ant taxa investigated so far either[15]. In addition, several transcripts were linked to two Hsp70 cofactors and potential complex partners: Hsp40 (DnaJ), BAG2, and the Hsp100 ClpB homolog (Table 1). While both Hsp40 and BAG2 greatly enhance the Hsp70 folding function[24,25], ClpB forms a complex with Hsp70/Hsp40 proteins that disaggregates and solubilizes denatured protein aggregates in an ATP-dependent manner[26]. This indicates that Hsp70 family folding activity is critical to cope with stresses in *Cataglyphis*. Altogether, these results confirm the importance of the folding activity of the hsp70 family to face adverse heat-shocks. Furthermore, whether directly via the HSF1 pathway or indirectly by heat-induced oxidative damages to the mitochondria[27], the joined induction of both *hsc70-5* and *hsp60* supports evidence of a major focus of the silver ant in safeguarding mitochondria integrity and energy production in response to higher temperatures.

The small heat shock proteins (sHsps) family was represented by two transcripts that matched up with the pro- tein Efl21 in *Drosophila* (encoded by *l(2)efl*), which is the ortholog of the Alpha-crystallin B chain in vertebrates (encoded by *CRYAB*)[28]. sHsps bind to and hold unfolded proteins in specific conformations, allowing folding machinery composed of other chaperones to operate[29]. In *D. melanogaster*, Efl21 stabilizes intermediate filament proteins and prevents them from aggregating under deleterious conditions, thus ensuring the structural integrity of the cytoskeleton, organelle morphology and the myofilaments[28]. Ants have three to six copies of *l(2)efl* that lack the putative HSEs[15] and accordingly, gene expression was not heat inducible in the two species tested so far, *A. picea* and *P. barbatus*. In contrast, our data indicate that expression of the two *l(2)efl* transcripts was strongly heat inducible in *C. bombycina*. Furthermore, three strongly expressed transcripts were each associated with specific proteins involved in muscle structure and function: muscle M-line assembly protein unc-89, two pore calcium channel protein 1 (TPC1), and nitric oxide synthase interacting protein (NOSIP). These three proteins are essen- tial for Ca^{2+} signaling during muscle contraction, and unc-89 is also involved in the assembly and organization of sarcomere[30–32]. This suggests that the HSR in *C. bombycina* also at least partially involves safeguarding muscle tissue organization. The Sahara silver ant is one of world's fastest running insects: its speed helps escape potential heat damage inflicted by ground temperatures of up to 70 °C[11,21]. Loss of muscle coordination would certainly mean death for foragers. Strong upregulation of *l(2)efl* might be a *Cataglyphis*-specific adaptation promoting worker survival.

Among heat-shock proteins, the Hsp90 family was represented by two transcripts that matched up with the Gp93 and Hsp83 protein. Heat-inducibility of *hsp83* was previously confirmed by qPCR in two species of *Cataglyphis*, including the silver ant[14]. Members of the Hsp90 family act as molecular chaperones, but they also work with a wide array of co-chaperones to regulate various biological pathways[33]. Accordingly, we found 6 tran- scripts directly coding for potential Hsp90 co-factors involved in signal transduction, and more specifically with the cell-cycle division (CDC37: 3 transcripts[34], and CHORDC1: 3 transcripts[35]). Three more transcripts matched up with signal transduction proteins also involved in regulating the cell cycle: 14-3-3 zeta[36], ANKLE2[37], and PPP2R2A[38]. Operating with the Hsp90-CDC37 co-chaperone complex, these three proteins are involved in the Akt/PkB signaling pathway that regulates cell proliferation, survival and apoptosis[39]. This finding was bolstered by the KEGG results, which revealed enrichment in the Akt/Pkb pathway (Fig. S7). The negative impacts of heat stress on mitotic activity are well known[40] and the HSR promotes survival by shutting down non-essential cellular processes while promoting macromolecular stability[41]. These results are consistent with a significant modulation of the cell-cycle and the ensuing rebalance of cellular resources.

As mentioned above, analysis of patterns of selection of 32 coding sequences among the 67 consistently and strongly expressed transcripts in response to heat stress indicated they were largely dominated by purifying selec- tion. However, among four genes for which sequences were investigated for a sufficient number of other ant species, a more detailed analysis suggested that for two of them (*hsc70-4 h2* and *hsc70-5*), positive selection had occurred more often along the *Cataglyphis* lineage than along the remaining branches of the tree. Even though its coding sequence might not be under positive selection, it is possible that the specific heat-inducibility of a gene (as observed in *hsc70-5*, *hsp-60 mitochondrial*, *l(2)efl*) has evolved through modifications of its promoter region and structure of its HSE[8]. Such promoter regions may evolve quite differently in response to habitat conditions. For example, in the diptera *Stratiomys singularior*, which lives in thermally variable and chemically aggressive and hypersaline conditions, all five *hsp70* genes have different promoter regions with a unique pattern of HSE, while in the relative *Oxycera pardalina* inhabiting cold springs, all *hsp70* genes have identical promoters[42]. Given the variability of HSEs sites, determining the exact sequence and structure of *hsps* genes' promoter regions in *C. bombycina* and comparing them to those of other related ants would be crucial to further understand the pattern of induction observed in the silver ant and the evolution of its physiological response to heat-stress.

The differential heat-inducibility of HSPs highlighted in this study could represent key adaptations to tolerate short-term and extreme thermal regime. Because triggering of the HSR is energetically costly as stress increases in frequency[43], alternative stress-resistance mechanisms involving structural changes are likely to be selected for long-term shifts in thermal performances[44]. Examples of such mechanisms are common in extremophile organisms; they include structural transitions in thermal optimum of proteins, higher temperature threshold for triggering the HSR, or modification of biological membrane composition to adapt fluidity to novel ther- mal regimes[10,44–46]. Signs of increased positive selection of *hsc70-4 h2* and *hsc70-5* molecular chaperones in *Cataglyphis* may indicate that those evolutionary mechanisms for thermal resistance are at work. This premise is supported by previous studies on two northern American *Aphaenogaster* species where such structural changes, rather than an enhanced HSR, are likely responsible for the increase in upper thermal tolerance of *A. carolinensis*'s

as compared to its more mesophilic relative *A. picea*[47]. However, heat-induction of *hsps* in these two species was still correlated with punctual workers acclimation to higher temperatures[18], as is the case in *Cataglyphis*. Most ants actively adapt depth and architecture of their nest to best match their own thermal optimum[48], and trig- gering the HSR might only be required when foragers exit the nest. In the Saharan silver ant both mechanisms likely co-occur to allow workers to seek food in the desert. Structural changes for long-term thermal resistance, complemented by constitutive production of Hsc70-3/Hsc70-4[13,14] and transient production of Hsp70 co-factors, mitochondrial Hsps and small Hsps, might be the best balance between the need for a swift cellular response when foragers burst out the nest and maintenance cost of molecular chaperones. A larger scale, point-to-point comparison between heat-tolerant ant species and their mesophilic relatives would be needed to validate this scenario and unravel the evolutionary mechanisms leading to thermal scavenging in ants.

Our study highlights a specific heat-induction of several heat-shock proteins that hasn't been reported in ant taxa so far (*hsc70-5*, *hsp60*, *hsp20*), and an increased level of positive selection in the *Cataglyphis* lineage for *hsc70-4 h2* and *hsc70-5*. This suggests that the heat-shock response of this thermal scavenger provides enhanced support to mitochondrial function and muscular tissue integrity, likely reflecting the increased need for this insect for proper motor function to face the intense stress from foraging at high speed on the burning ground. Such adaptations could give *C. bombycina* a much-needed edge in surviving the scorching heat of the Sahara Desert.

Methods

Field sampling and laboratory rearing. Fifteen colonies of *C. bombycina* were collected near Zagora (30°19′56″North; 5°50′18″West), in the Draa Valley of southern Morocco in early May 2015. They were reared under constant environmental conditions (25 °C, 60% relative humidity, 12:12 light-dark cycle) and fed sugar solution *ad libitum* and sliced mealworms twice a week. The colonies spent at least two months under these conditions to decrease pre-collection environmental influences before the experiment took place, which only used workers born and raised in the laboratory (*i.e.*, from the egg to the adult stage). Belgium does not have eth- ical requirements concerning work with ants, and experiments were carried out in accordance with the relevant guidelines and regulations.

Heat stress experiment. We used the experimental methodology described in Willot *et al.*[14]. For a given colony, 20 randomly chosen workers were selected to form 2 groups of 10 workers each separated in 50-ml glass vial containing a moist cotton ball. One group was kept at 25 °C (control treatment), and the other exposed to 45 °C (heat-stress treatment), both inside their vial submerged in a digitally controlled water bath for three hours. This procedure was replicated 4 times in 4 different colonies to obtain 4 controls and 4 heat-shocked replicates. The temperature inside the vials was monitored using 0.075-mm-diameter thermocouples connected to a digital thermometer. In *C. bombycina*, this duration of heat exposure induces a HSR without causing acute mortality[14,15].

RNA-seq library preparation and Illumina sequencing. The whole bodies of control and heat-stressed ants were homogenized for three minutes at maximum speed in a mixer mill using 2.8-mm zirconium oxide beads. Total RNA was extracted using TRIzol reagent in accordance with the manufacturer's instructions. RNA was quantified with an ARN Quant-iT RiboGreen Kit (ThermoFisher Scientific, CA, USA); the samples were then sent to a sequencing facility (GenoScreen, Lille, France). RNA libraries were generated using paired-end sequencing implemented by an Illumina HiSeq 2500 system in high-output mode; read length was 100 bp. After quality filtering, the mean number of reads per sample was 21.97 M (range: 18.40–25.08 M).

De novo transcriptome assembly, transcript mapping, and identification of heat-inducible genes. To generate the reference assembly, the sequenced reads for all the samples were first combined and then assembled, using the Trinotate annotation suite (*i.e.*, Trinity software; trinityrnaseq 2.2.0). Subsequently, the reads for each sample were independently mapped back onto this reference assembly, and all the transcripts were quantified using RSEM (RSEM v1.3.0)[49]. To determine which transcripts were differentially expressed in control versus heat-stressed ants, expression levels were quantified using edgeR (edgeR 3.18.1)[50].

The edgeR model was constructed using a single pairwise comparison between two groups (HS vs NHS). The dispersion was estimated using the qCML method (estimateDisp). Differential expression between the two groups was performed using the quasi-likelihood (QL) method and a QL F-test (glmQLFit, glmQLFTest). Transcripts with a greater than background level of expression (mean log CPM > 0) and a low false discovery rate (FDR < 0.05) were used in the downstream analysis below.

Gene ontology, functional annotation, and KEGG annotation. To understand the biological signifi- cance of the genes displaying heat-induced expression, we investigated (i) gene ontology (GO) (*i.e.*, detailed anno- tations of gene function, related biological processes, and gene product cell locations) and (ii) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps (*i.e.*, annotations of gene metabolic and cellular functions).

First, the transcripts were searched against NCBI's non-redundant (nr) protein database using BLASTX. The search was restricted to arthropods and employed an e-value cut-off of $10e^{-5}$. Transcripts were annotated with GO terms using BLAST2GO program[51] and an e-value cut-off of $10e^{-5}$. A second layer of GO terms was added to the transcripts using InterProScan online[52], and WEGO software[53] was used to functionally classify the terms. Second, the transcripts were annotated for biochemical pathways[54] using the KEGG Automatic Annotation Server (KAAS) for ortholog assignment and pathway mapping[55].

DNA sequence variation analyses to detect patterns of selection. Among all isolated heat-induced transcripts, we retained 32 sequences for which we could identify an orthologous copy in the annotated genome of the closely related ant *Camponotus floridanus*. We isolated the coding sequence (CDS) of each one of these transcripts and conducted for each of them a classic d_N/d_S test[56] on the alignment of the sequences from both

species, using the program codeml (package PAML version 4.8[57]). This test has the ability to highlight an overall pattern of negative or positive selection for a protein coding gene, by identifying a deficit or excess of non-synonymous mutations compared to expectations under a hypothesis of neutral evolution[58]. In addition, for 4 transcripts for which we found orthologous sequences in multiple ant species (see Supplementary Material for a list of species used), we conducted a likelihood ratio test to detect positive selection[59], also using codeml. In these cases, we compared two codon-substitution models, one that assumes a single d_N/d_S ratio across the entire ant phylogenetic tree, with another that assumes two different d_N/d_S ratios: one for the branch leading to the genus *Cataglyphis* and another for all other branches of the tree. Another version of the two-ratio model was also created by assuming the same ratio for the branch leading to the genus *Cataglyphis* and for all branches within this genus. A likelihood ratio test was conducted to determine whether the lineage leading to Cataglyphis, possibly along with the branches within the Cataglyphis clade, is (are) characterized by a larger d_N/d_S ratio than the remaining lineages of the tree. The likelihood of the ant phylogenetic tree[60] (we used a simplified tree that included only the species for which sequences were included in the analysis, see Table S2) was computed under both the one-ratio and two-ratio models, and the two values were compared. We tested whether the two-ratio model fitted the data significantly better than the one-ratio model by comparing twice the log likelihood difference with a χ^2 distribu- tion (df = 1).

Data availability. The raw transcriptomic data analyzed during the current study have been submit- ted to NCBI's sequence read archive (https://www.ncbi.nlm.nih.gov/bioproject/419094) under accession no. PRJNA419094.

References

1. Feder, M. E. & Hofmann, G. E. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243–282 (1999).
2. Richter, K., Haslbeck, M. & Buchner, J. The heat shock response: Life on the verge of death. *Mol. Cell* **40**, 253–266 (2010).
3. Lindquist, S. Translational efficiency of heat-induced messages in *Drosophila melanogaster* cells. *J. Mol. Biol.* **137**, 151–158 (1980).
4. Parsell, D. & Lindquist, S. The function of heat-shock proteins in stress tolerance - degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437–496 (1993).
5. Courgeon, A., Maisonhaute, C. & Best-Belpomme, M. Heat shock proteins are induced by cadmium in *Drosophila* cells. *Exp. Cell Res.* **153**, 515–521 (1984).
6. Wheeler, J., Bieschke, E. & Tower, J. Muscle-specific expression of *Drosophila* hsp70 in response to aging and oxidative stress. *Proc. Natl. Acad. Sci. USA* 92, 10408–10412 (1995).
7. Duarte, F. *et al*. Transcription factors GAF and HSF act at distinct regulatory steps to modulate stress-induced gene activation. *Genes Dev.* **30**, 1731–1746 (2016).
8. Morimoto, R. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev.* **12**, 3788–3796 (1998).
9. Moseley, P. L. Heat shock proteins and heat adaptation of the whole organism. *J. Appl. Physiol.* **83**, 1413–1417 (1997).
10. Evgen'ev, M., Garbuz, D. & Zatsepina, O. G. Heat shock proteins and whole body adaptation to extreme environments. *Springer* (2014).
11. Wehner, R., Marsh, A. & Wehner, S. Desert ants on a thermal tightrope. *Nature* **357**, 586–587 (1992).
12. Hoffmann, A., Chown, S. & Clusella-Trullas, S. Upper thermal limits in terrestrial ectotherms: how constrained are they? *Funct. Ecol.* **27**, 934–949 (2012).
13. Gehring, W. & Wehner, R. Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara Desert. *Proc. Natl. Acad. Sci. USA* **92**, 2994–2998 (1995).
14. Willot, Q., Gueydan, C. & Aron, S. Proteome stability, heat hardening and heat-shock protein expression profiles in *Cataglyphis* desert ants. *J. Exp. Biol.* **220**, 1721–1728 (2017).
15. Nguyen, A., Gotelli, N. & Cahan, S. The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC Evol. Biol.* **16** (2016).
16. Helms Cahan, S. *et al*. Modulation of the heat shock response is associated with acclimation to novel temperatures but not adaptation to climatic variation in the ants *Aphaenogaster picea* and *A. rudis*. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **204**, 113–120 (2017).
17. Elekonich, M. Extreme thermotolerance and behavioral induction of 70-kDa heat shock proteins and their encoding genes in honey bees. *Cell Stress Chaperones* **14**, 219–226 (2008).
18. Santelli, E. *et al*. Structural Analysis of Siah1-Siah-interacting protein interactions and insights into the assembly of an E3 ligase multiprotein complex. *J. Biol. Chem.* **280**, 34278–34287 (2005).
19. Friant, S. Increased ubiquitin-dependent degradation can replace the essential requirement for heat shock protein induction. *EMBO J.* **22**, 3783–3791 (2003).
20. Wong, P. & Houry, W. Chaperone networks in bacteria: analysis of protein homeostasis in minimal cells. *J. Struct. Biol.* **146**, 79–89 (2004).
21. Boulay, R. *et al*. Social life in arid environments: the case study of *Cataglyphis* ants. *Annu. Rev. Entomol.* **62**, 305–321 (2017).
22. Zhu, J. *et al*. Knockdown of Hsc70-5/mortalin induces loss of synaptic mitochondria in a *Drosophila* Parkinson's disease model. *PLoS ONE* **8**, e83714 (2013).
23. Banerjee, S. & Chinthapalli, B. A proteomic screen with *Drosophila* Opa1-like identifies Hsc70-5/Mortalin as a regulator of mitochondrial morphology and cellular homeostasis. *Int. J. Biochem. Cell B* **54**, 36–48 (2014).
24. Fan, C., Lee, S. & Cyr, D. Mechanisms for regulation of Hsp70 function by Hsp40. *Cell Stress Chaperones* **8**, 309 (2003).
25. Qin, L., Guo, J., Zheng, Q. & Zhang, H. BAG2 structure, function and involvement in disease. *Cell. Mol. Biol. Lett.* **21** (2016).
26. Doyle, S. & Wickner, S. Hsp104 and ClpB: protein disaggregating machines. *Trends Biochem. Sci.* **34**, 40–48 (2009).
27. Belhadj Slimen, I. *et al*. Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. *Int. J. Hyperthermia* **30**, 513–523 (2014).
28. Wojtowicz, I. *et al*. Drosophila small heat shock protein CryAB ensures structural integrity of developing muscles, and proper muscle and heart performance. *Development* **142**, 994–1005 (2015).
29. Lee, G. A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. *EMBO J.* **16**, 659–671 (1997).
30. Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J. & Cohen, R. A. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**, 850–853 (1994).
31. Zhu, M., Evans, M., Ma, J., Parrington, J. & Galione, A. Two-pore channels for integrative Ca2+signaling. *Commun Integr Biol* **3**, 12–17 (2010).

32. Spooner, P., Bonner, J., Maricq, A., Benian, G. & Norman, K. Large isoforms of UNC-89 (Obscurin) are required for muscle cell architecture and optimal calcium release in *Caenorhabditis elegans*. *PLoS ONE* **7**, e40182 (2012).
33. Schwenkert, S., Hugel, T. & Cox, M. The Hsp90 ensemble: coordinated Hsp90–cochaperone complexes regulate diverse cellular processes. *Nat. Struct. Mol. Biol.* **21**, 1017–1021 (2014).
34. Hunter, T. & Poon, R. Cdc37: a protein kinase chaperone? *Trends Cell Biol.* **7**, 157–161 (1997).
35. Gano, J. & Simon, J. A. Proteomic investigation of ligand-dependent HSP90 complexes reveals CHORDC1 as a novel ADP- dependent HSP90-interacting protein. *Mol. Cell. Proteomics* **9**, 255–270 (2009).
36. Dalal, S., Yaffe, M. & DeCaprio, J. 14-3-3 family members act coordinately to regulate mitotic progression. *Cell Cycle* **3**, 670–675 (2004).
37. Asencio, C. *et al*. Coordination of kinase and phosphatase activities by Lem4 enables nuclear envelope reassembly during mitosis. *Cell* **150**, 122–135 (2012).
38. Janssens, V. & Goris, J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* **353**, 417 (2001).
39. Song, G., Ouyang, G. & Bao, S. The activation of Akt/PKB signaling pathway and cell survival. *J. Cell. Mol. Med.* **9**, 59–71 (2005).
40. Maldonado-Codina, G., Llamazares, S. & Glover, D. M. Heat shock results in cell cycle delay and synchronisation of mitotic domains in cellularised *Drosophila melanogaster* embryos. *J. Cell. Sci.* **105**, 711–720 (1993).
41. Nover, L., Hightower, L. & Hightower, L. *Heat shock and development*. (Springer, 1991).
42. Garbuz, D. G. *et al*. Organization and evolution of hsp70 clusters strikingly differ in two species of *Stratiomyidae* (Diptera) inhabiting thermally contrasting environments. *BMC Evol. Biol*. **11** (2011).
43. Krebs, R. & Feder, M. Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *J. Insect Physiol.* **44**, 1091–1101 (1998).
44. Stetter, K. Extremophiles and their adaptation to hot environments. *FEBS Letters* **452**, 22–25 (1999).
45. Tomanek, L. & Somero, G. Variation in the heat shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **124**, 107 (1999).
46. Zatsepina, O. G. *et al*. Thermotolerant desert lizards characteristically differ in terms of heat-shock system regulation. *J. Exp. Biol.* **203**, 1017–1025 (2000).
47. Stanton-Geddes, J. *et al*. Thermal reactionomes reveal divergent responses to thermal extremes in warm and cool-climate ant species. *BMC Genomics* **17** (2016).
48. Hölldobler, B. & Wilson, E. O. The ants. *Harvard University Press* (1990).
49. Li, B. & Dewey, C. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).
50. Robinson, M., McCarthy, D. & Smyth, G. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2009).
51. Conesa, A. *et al*. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674–3676 (2005).
52. Jones, P. *et al*. InterProScan 5: genome-scale protein function classification. *Bioinformatics* **30**, 1236–1240 (2014).
53. Ye, J. *et al*. WEGO: a web tool for plotting GO annotations. *Nucleic Acids Res.* **34**, W293–W297 (2006).
54. Kanehisa, M. & Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
55. Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A. & Kanehisa, M. KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res.* **35**, W182–W185 (2007).
56. Nei, M. & Gojobori, T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* **3**, 418–426 (1986).
57. Yang, Z. PAML4: a program package for phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24**, 1586–1591 (2007).
58. Nielsen, R. Molecular signatures of natural selection. *Annu. Rev. Genet.* **39**, 197–218 (2005).
59. Yang, Z. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Mol. Biol. Evol.* **15**, 568–573 (1998).
60. Moreau, C. & Bell, C. Testing the museum versus cradle tropical biological diversity hypothesis: phylogeny, diversification, and ancestral biogeographic range evolution of the ants. *Evolution* **67**, 2240–2257 (2013).

Acknowledgements

This work was supported by a Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture (FRIA) scholarship (to Q.W.) and CDR funding (to S.A.; J.0151.16) from the Belgian Fonds de la Recherche Scientifique (FRS-FNRS).

Author Contributions

Q.W., C.G. and S.A. conceived and planned the study. Q.W. and S.A. collected samples. Q.W., P.M. and M.D. performed molecular work and analyzed the data. All authors contributed to drafting the article, approved the final published version and agreed to be held accountable for all aspects of the work.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-27628-2.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Gene	dN/dS
14-3-3 Zeta	0.001
ankyrin	0.218
asparagine synthetase	0.0893
BAG2	0.111
calcium binding mt carrier	0.0942
calcyclin binding protein	0.0647
Chromodomain helicase DNA binding protein	0.05
Caseinolytic Peptidase B	0.1112
cystein-rich protein 2-binding protein 1	0.1457
cystein histidine-rich domain-containing protein	0.1489
dnaJ homolog subfamily A member 1	0.0211
endoplasmic reticulum metallopeptidase 1	0.1616
endoplasmin	0.0787
eukaryotic translation initiation factor 1A	0.00726
exonuclease mut-7 homolog	0.1443
Hsc70-3	0.0256
nitric oxide synthase-interacting protein	0.0467
nodal modulator 1	0.1272
phospholipase DDHD1	0.0747
protein phosphatase PP2A	0.0147
RNA polymerase gld-2	0.2885
SZT2	0.0937
transmembrane protein 120	0.0678
two pore calcium channel protein 1	0.0857
muscle M-line assembly protein unc-89	0.0989
uncharacterized LOC105258422	0.037
WD repeat-containing protein 89	0.1553

Table S1. *dN/dS* ratios calculated for 28 CDS among the 67 consistently heat-induced transcripts between C. bombycina and the closely related ant *Camponotus floridanus*.

Gene	*hsc70-4 h1*		*hsc70-4 h2*		*hsc70-5*		*hsp83*	
	d_N/d_S	Ln likelihood	d_N/d_S	Ln likelihood	d_N/d_S	Ln likelihood	d_N/d_S	Ln likelihood
Single d_N/d_S (Ha)	0.004	-5002.49	0.018	-5251.95	0.025	-4561.78	0.014	-7326.76
Cataglyphis lineage (Hb)	15.965	-5013.64	0.059	-5248.54	0.0491	-4560.71	0.016	-7326.75
Other ant lineages	0.004		0.016		0.024		0.014	
Cataglyphis lineage and clade (Hc)	0.001	-5001.49	0.036	-5250.44	0.052	-4559.67	0.009	-7326.59
Other ant lineages	0.004		0.016		0.023		0.014	
	Ratio	Significance	Ratio	Significance	Ratio	Significance	Ratio	Significance
Ha/Hb	L(Hb) < L (Ha)	n.s.	6.82	**	2.14	n.s.	0.02	n.s.
Ha/Hc	2	n.s.	3.02	n.s.	4.22	*	0.34	n.s.

Table S2. Likelihood ratio test evaluating whether the lineage leading to *Cataglyphis* (model B) or this lineage and the branches within the *Cataglyphis* genus (model C) are characterized by a larger dN/dS ratio than other branches in the ant tree (*:p<0.05, **: p<0.01). Species included in this test are *Acromyrmex echinatior*, *Atta cephalotes*, *Pogonomyrmex barbatus*, *Solenopsis invicta*, *Cardiocondya obscurior*, *Linepithema humile*, *Camponotus floridanus*, *Formica exsecta*, *Cataglyphis hispanica* and *Cataglyphis bombycina*.

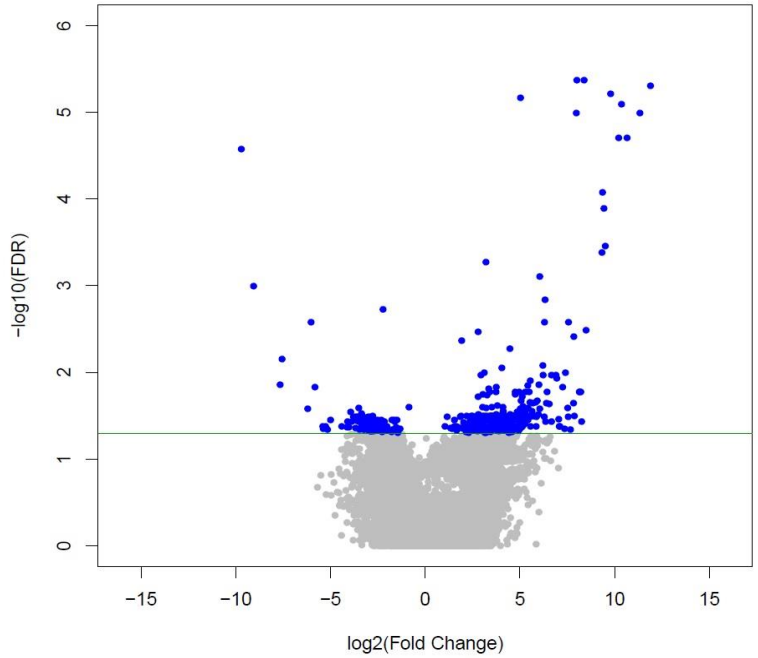

Figure S1. Relationship between fold change (FC) and false discovery rate (FDR) of the differentially expressed transcripts in response to heat-stress in the ant *C. bombycina*. The 533 transcripts with above background levels of expression and a FDR inferior to 0.05 (above green line) were selected for further screening.

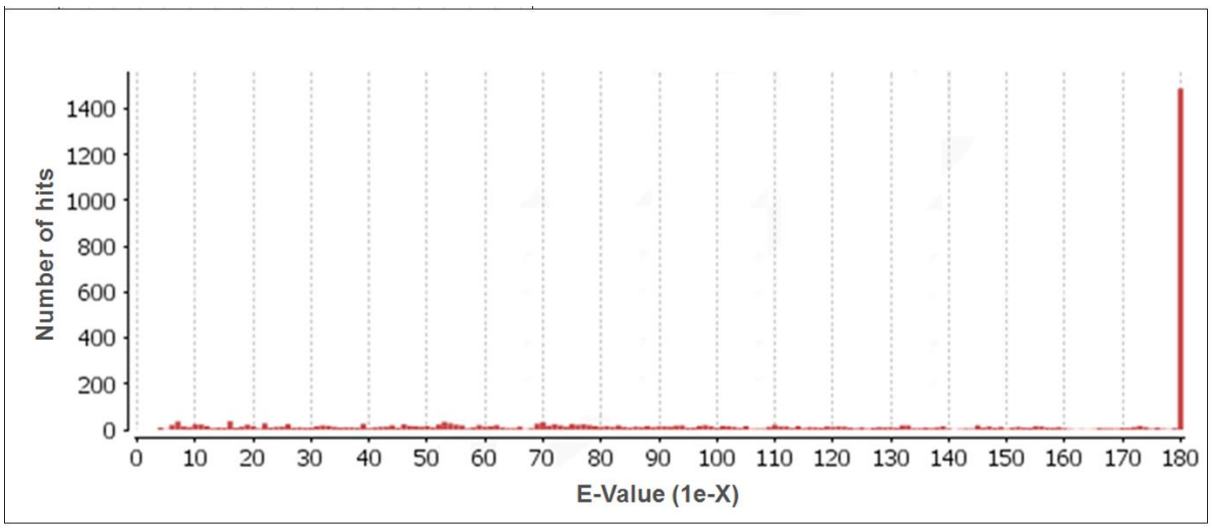

Figure S2. Distribution of the e-values for the 533 transcripts displaying differential expression in response to heat stress in *C. bombycina*. The e-values were obtained when the transcripts were queried against the NCBI non-redundant protein database (arthropod records only) using BLASTX. Most e-values were equal to or less than $1e^{-180}$, indicating transcript annotation was reliable.

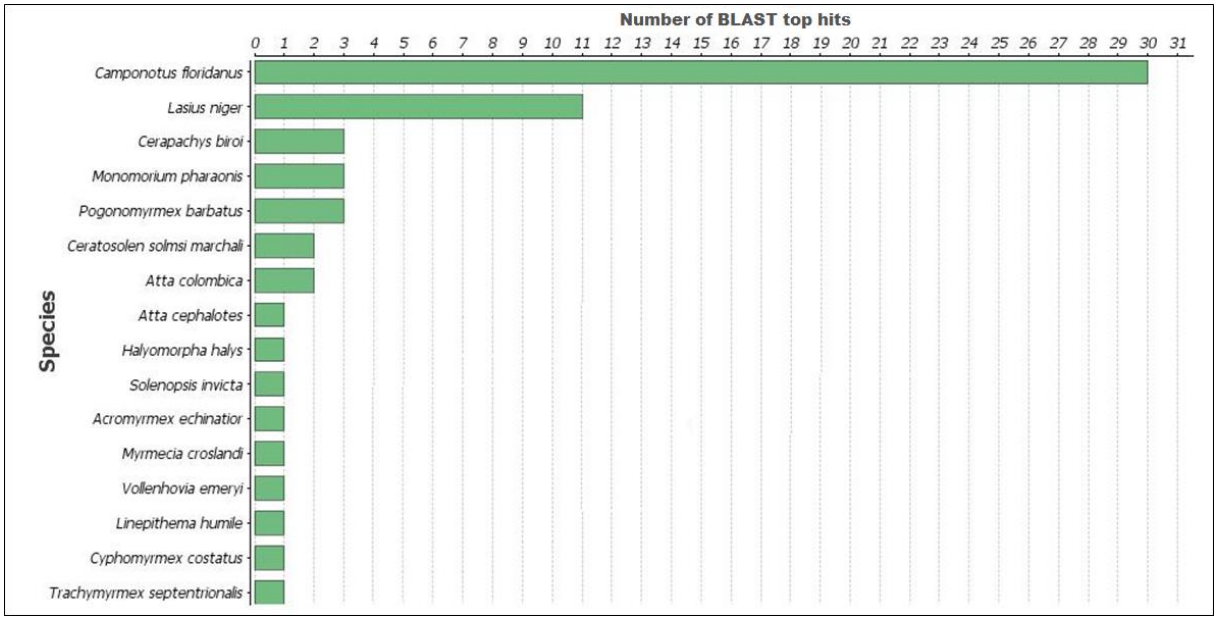

Figure S3. Distribution of best transcript hits with proteins from various insect species. Sixty-seven transcripts showing strong and consistent heat-induced expression (FC > 2 and RSD < 0.4) were queried against the NCBI non-redundant protein database (arthropod records only) using BLASTX (<10e^{-5}). The most represented taxa for the best hit of each match were mainly other ant species.

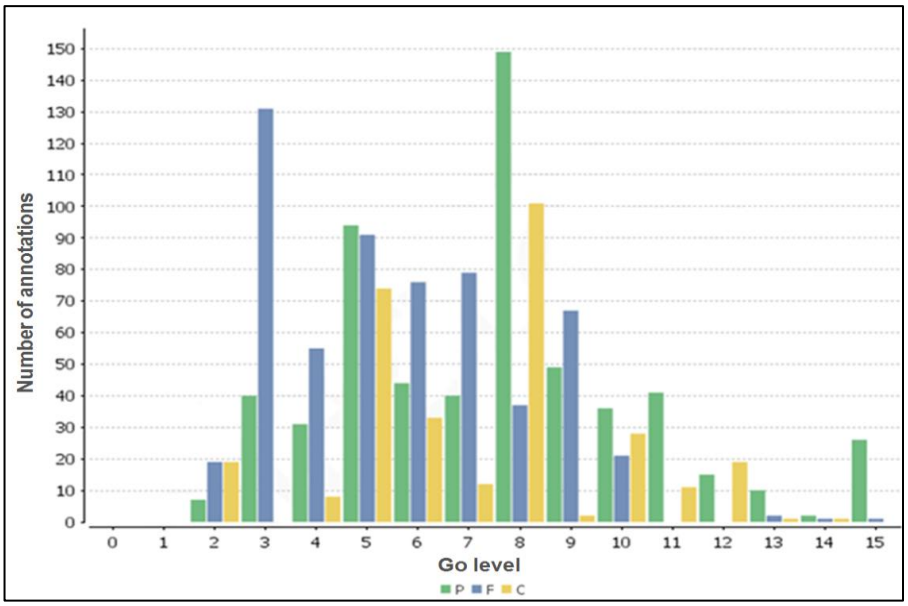

Figure S4. Distribution of GO levels for the 393 annotated transcripts. The transcripts were distributed across the three GO-classification domains: cellular component (GO levels 5–8), biological process (GO levels 4–8), and molecular function (GO levels 3–5). There were 1,481 annotations in total, and the mean annotation level was 6.7 (SD = 2.8).

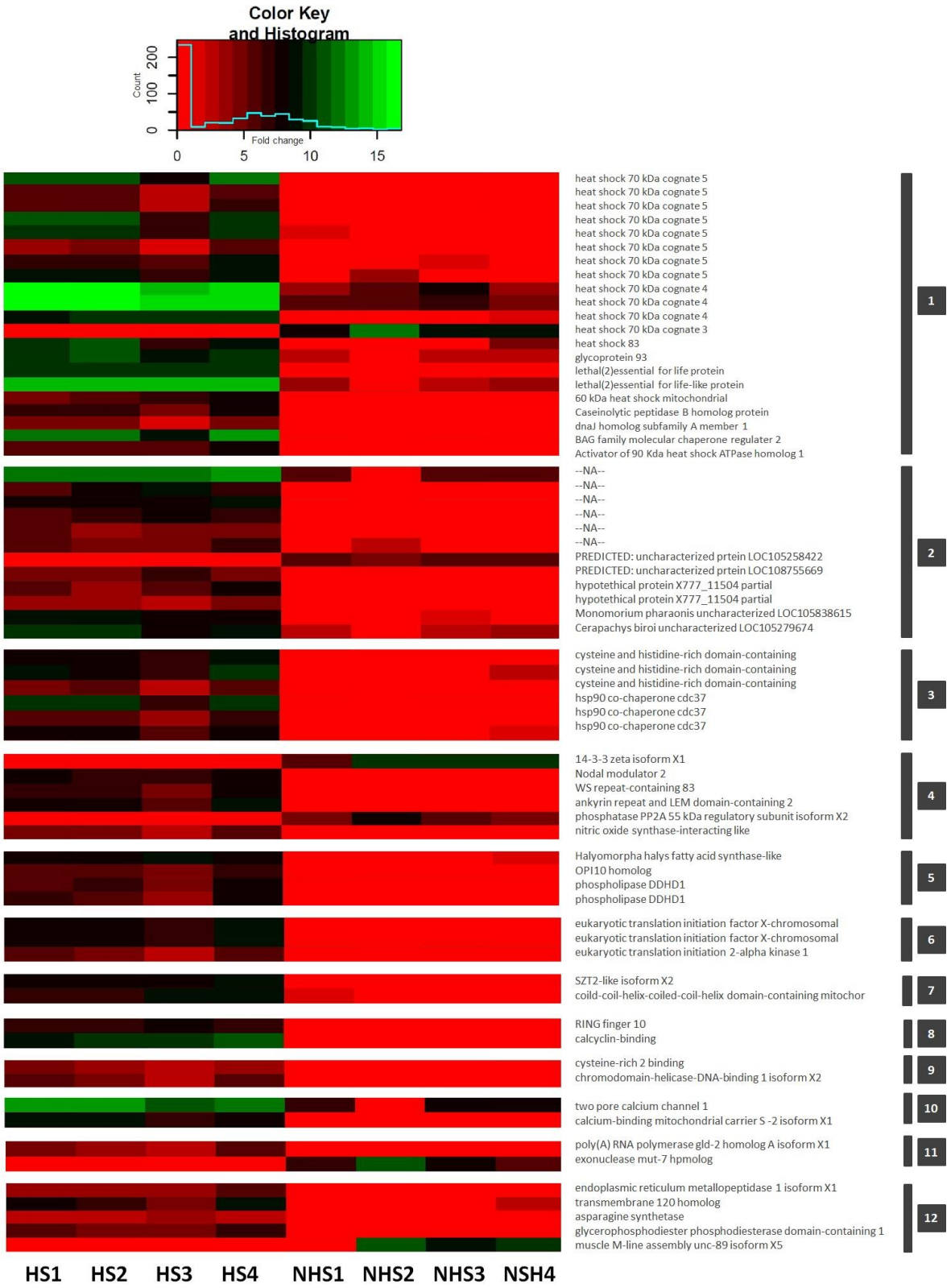

Figure S5. Color-coded relative expression levels and distribution curve of the 67 strongly and consistently expressed transcripts. The numeric codes signify the following: **1**: heat-shock proteins and co-chaperones, **2**: no match, **3**: Hsp90 co-chaperones, **4**: cell signal transduction proteins, **5**: lipid metabolic proteins, **6**: translation initiation factors, **7**: chromatin-remodeling proteins, **8**: proteins regulating oxidative stress, **9**: proteins in the ubiquitin-proteasome degradation pathway, **10**: calcium transport proteins, **11**: RNA-modifying proteins, and **12**: other protein types. **HS**: heat-stress group; **NHS**: control group.

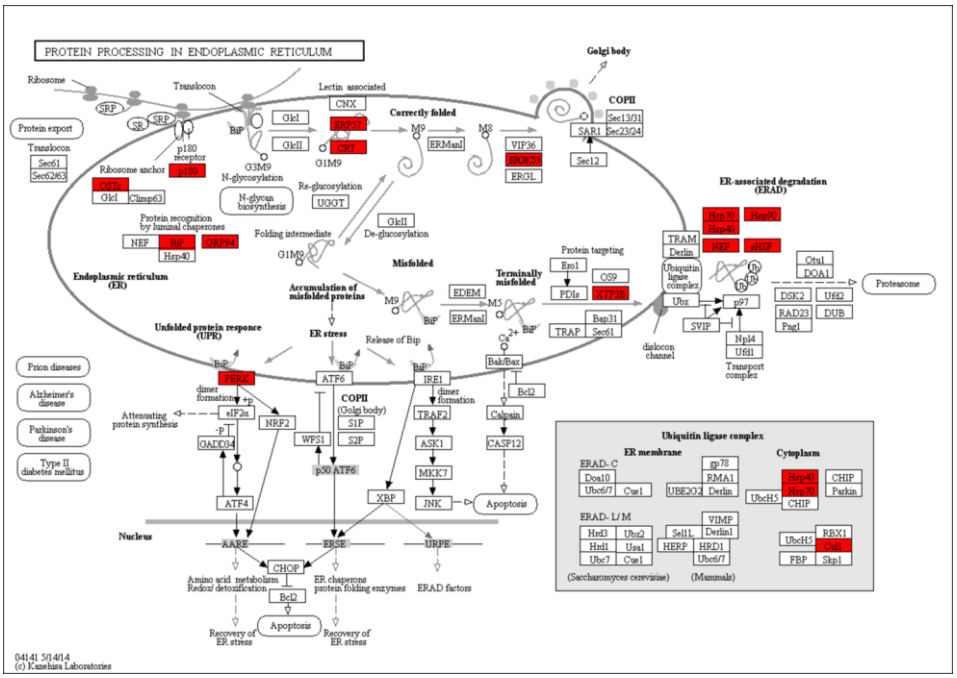

Figure S6. KEGG annotation pathway for transcripts involved in protein processing in the endoplasmic reticulum (map04141). The 18 positive hits are colored in red; the transcripts matched up with proteins involved in protein folding, translocation, and degradation.

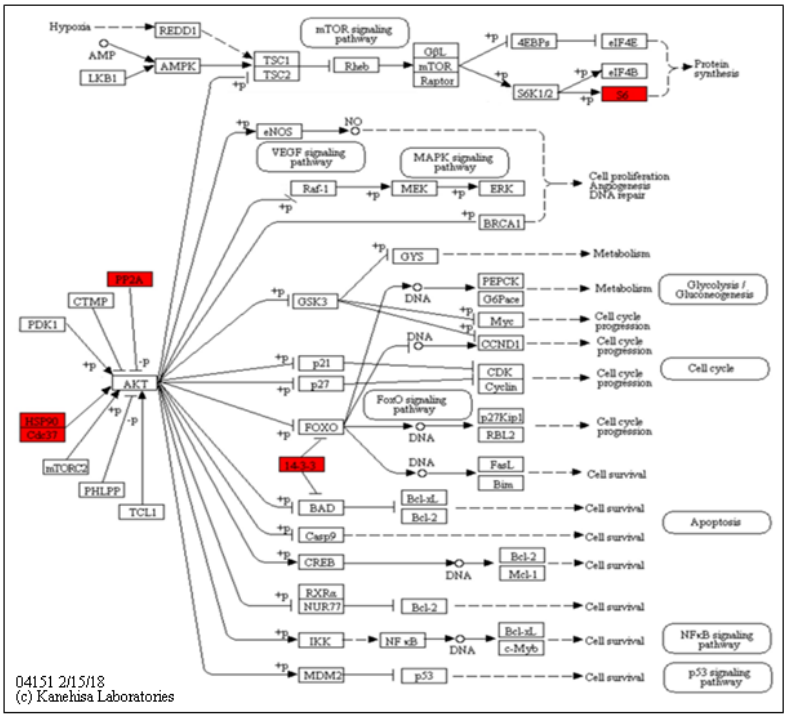

Figure S7. KEGG annotation pathway for transcripts involved in the cell cycle control via the AKT pathway (modified from map04151). Positive hits are colored in red. There is enrichment in transcripts linked with the cell cycle control and apoptosis.

Chapter IV

Project: Parallel molecular evolution of thermal tolerance in desert ants

Quentin Willot, Remy Perez, Matthieu Defrance, & Serge Aron

Objective

This chapter investigates survival curves and heat-tolerance mechanisms in *Ocymyrmex robustior*, using RNA-sequencing, western blotting and survival assays. The aim of this project is to integrate these data inside a larger comparative framkework of thermal tolerance mechanisms in thermal scavenging ants. By doing so, we will be able to identify the trends of convergent evolutive pressures on physiology to support thermal scavenging. This chapter takes support from chapters I, II, and III, for it demands an extensive knowledge of both physiology and heat-budgets of species, that themselves are tightly linked to behavior, morphology, and species environment.

Abstract

A few genera of desert ants rely on the high thermal hardiness of workers to scavenge for the corpses of heat-stricken arthropods. They are among the most heat-tolerant land animals known so far and, although crucial to their ecology, little is known about the physiological adaptations developed by those insects to tolerate elevated body temperatures. Here, we studied the heat-shock response of the ant *Ocymyrmex robustior* from the Namib Desert, one of the world's driest and hottest regions. We first performed a differential gene expression analysis between control and heat-stressed individuals using RNA-seq and second, we determined the functional consequences of heat stress by assessing their survival rate, heat hardening, and Hsp70 expression. We show that, similar to the Sahara silver ant *Cataglyphis bombycina*, *O. robustior* constitutively express high levels of Hsp70s, with limited subsequent heat-induction of the proteins, and limited heat-hardening capabilities. Furthermore, while some processes involved in protecting sensitive aspects of cellular homeostasis are similar in both species, we show that the exact genes involved as well as their relative expression varies between the two. Overall, our results highlight a parallel evolution of the molecular mechanisms aimed at promoting individual survival to heat-stress, but the exact means of doing so varies according to the species evolutive history.

Introduction

The ability to rely on thermal tolerance to exploit temperatures unbearable for competitors or predators is described so far in a few desert ant genera only. Those genera include the *Cataglyphis* genus from north Africa and the Mediterranean Basin, the *Ocymyrmex* genus from the Namib and Karoo deserts of southern Africa, and the *Melophorus* genus in the Australian outback. Workers of those species actively exploit their high thermal hardiness to seek for arthropod corpses in plain day[1,2,3], a behavior referred to as thermal scavenging. This implies that workers themselves need to survive the scorching heat and accordingly, thermal scavenging ants share the highest critical thermal maximums (CT_{max}) known in land animals so far (*Ocymyrmex robustior*, 51.2°C; *Cataglyphis bombycina*, 53.6°C; *Melophorus bagoti*, 56.6°C) [1-5]. These three ant genera are phylogenetically distant; *Ocymyrmex* was estimated to have diverged from the others around 130 Mi years ago while *Cataglyphis* and *Melophorus* diverged around 85 Mi years ago[6]. Yet, they share similar behavioral and morphological traits to cope with intense and transient bursts of heat-stresses coming with thermal scavenging. While running on the burning ground, foragers actively exploit thermal refuges to intermittently cool themselves[1-5]. From a morphological perspective, they have slender appearances and possess long-legs that elevate their body away from the ground. They display fast running speed, which cool their body by a convective heat-dissipative effect while seeking for food[7]. From a physiological perspective, however, the current knowledge surrounding their thermal tolerance mechanisms remains scarce and mostly concentrates on the Sahara ant *C. bombycina* so far. It has been shown that, contrasting with mesophilic ants, *C. bombycina* workers do accumulate heat-shock protein 70s before emerging from the nest, thereby bypassing the need to acclimate to sudden heat exposure[8,9]. In response to heat-stress, worker express a wide array of genes that are involved with molecular chaperoning, muscular functions, and mitochondrial protection[10]. This likely depicts the higher requirement for motor skills that *C. bombycina* needs to meet during its foraging activity at high velocity and under intense heat-pressure.

Here, we aimed to place our knowledge of the physiological mechanisms responsible for the elevated thermal tolerance of thermal scavenging ants in a comparative framework. For this purpose, we used the ant *Ocymyrmex robustior* as a model. *O. robustior* dwells in the gravel plain of the Namib Desert and display a typical thermal scavenging behavior that is similar to the Saharan silver ant. We first performed heat-tolerance experiments and investigated the potential effect of heat-hardening (*i.e* an acquired resistance to heat-stress through acclimation) on worker survival under laboratory conditions. Second, we assessed the expression in workers of Hsc70 before and after heat-stress, using western blot. Third, we analyzed the species' transcriptomic response to heat-stress using RNA-sequencing. We compared our data to those previously acquired for the Saharan ant *C. bombycina*. Our results highlight that mechanisms shifting thermal tolerance up have been functionally selected in both species and promote heat-stress survival. However, details of the molecular pathways responsible for this feat varies with species history, underpinning that parallel evolution of heat-tolerance is not a one-way road and can take several different paths leading to similar heat-hardiness.

Methods

Field sampling and laboratory rearing

Colonies of *Ocymyrmex robustior* were collected in the Kuiseb River bed, near the Gobabeb research and training center, Erongo, Namibia (23°33'42'' South; 15°2'29'' East). They were reared under constant environmental conditions (25°C, 60% relative humidity, 12:12 light-dark cycle) and fed sugar solution *ad libitum* and sliced mealworms twice a week. The colonies spent at least two months under these conditions to decrease pre-collection environmental influences before the experiment took place, which only used workers born and raised in the laboratory (*i.e.,* from the egg to the adult stage). Belgium does not have ethical requirements concerning work with ants, and experiments were carried out in accordance with the relevant guidelines and regulations.

Thermal tolerance and heat hardening

General thermal tolerance was determined using heat stress experiments according to the work of Willot *et al.*,2017[9]. From each colony, 20 randomly chosen workers were selected to form 2 groups of 10 workers each separated in 50-ml glass vial containing a moist cotton ball. One group was kept at 25°C (control treatment) and the other exposed to a stable heat-regime for three hours inside their vial submerged in a digitally controlled water bath (42, 45,47 or 49°C). This procedure was replicated 3 times per stress temperature. The temperature inside the vials was monitored using 0.075-mm-diameter thermocouples connected to a digital thermometer. Heat hardening was assessed by pre-exposing workers to mild heat stress (37°C) for 2 hours before they experienced severe heat stress (47°C) for 3 hours. We used worker survival rates as proxies for levels of thermal tolerance and heat hardening. Survival rates in the thermal-tolerance and heat-hardening experiments were compared using one-way analyses of variance (ANOVAs). Multiple comparisons among pairs of means were carried out using Tukey *post-hoc* tests.

Constitutive and induced levels of Hsc70s

Constitutive levels of Hsc70s were estimated using western blot. We extracted total protein from 20 mg of randomly selected control (25°C) and heat-shocked (45°C) workers. We also used *Drosophila melanogaster* as an outgroup control, and 20 mg of total proteins were extracted from cell cultures (Schneider 2 cell line) maintained either in control (27°C) or heat-shocked (37°C) conditions. Protein concentrations were determined using the Bradford procedure (BioRad, CA, USA). Fifteen micrograms of the extract were mixed with 6-fold concentrated Laemmli buffer (1% SDS, 5%β-mercaptoethanol, and 10% glycerin; Sigma-Aldrich Chemie GmbH, Germany) in a 1:6 ratio. The samples were kept at 95°C for 5 minutes. They were then diluted in Laemmli buffer and analyzed using SDS-PAGE. The total signal for each lane was quantified using a CCD camera, which established the control loading values for each sample. Second, the gel was electrotransferred onto an Amersham Protran 0.45 nitrocellulose blotting membrane (GE Healthcare, Life Sciences, UK). Skim milk (1.5%) was used for blocking (1h, 20°C) and to dilute the antibodies. The membrane was washed three times with TBS-T buffer (10 mM Tris, 150 mM NaCl, and 2% Tween-20) and incubated overnight at 4°C with an anti-HSP70 monoclonal mouse antibody (1/2000 dilution; clone BRM-22, Sigma-Aldrich, Israel) and an Anti-histone H3 polyclonal rabbit antibody (1/2000 dilution; 07-690 Milipore). Then, secondary antibodies (1/10000 dilution; anti-mouse IgG peroxidase, anti-rabbit IgG peroxidase, Sigma-Aldrich, Israel) were added, and the solution was left at 20°C for 2 hours. Signal strength was quantified using an Odyssey® FC Imaging System (Li-Cor® Bioscience, NE, USA).

RNA-seq library preparation and Illumina sequencing

The whole bodies of control and heat-stressed ants were homogenized for three minutes at maximum speed in a mixer mill using 2.8-mm zirconium oxide beads. Total RNA was extracted using TRIzol reagent in accordance with the manufacturer's instructions. RNA was quantified with an ARN Quant-iT™ RiboGreen® Kit (ThermoFisher Scientific, CA, USA); the samples were then sent to a sequencing facility (BGI, Honk Kong). RNA libraries for *de novo* assembly were generated using paired-end sequencing implemented by an Illumina HiSeq 2500 system in high-output mode; read length was 100 bp. RNA libraries for the differential gene expression analysis (DGE) to heat were generated using single-end sequencing implemented by an Illumina HiSeq 2500 system in high-output mode; read length was 50 bp.

De novo transcriptome assembly, transcript mapping, and identification of heat-inducible genes

The *de novo* reference assembly was generated using the Trinotate annotation suite (*i.e.*, Trinity software; trinityrnaseq 2.2.0). Subsequently, the reads for each sample of the DGE were independently mapped back onto this reference assembly, and all the transcripts were quantified using RSEM (RSEM v1.3.0)[11]. To determine which transcripts were differentially expressed in control versus heat-stressed ants, expression levels were quantified using edgeR (edgeR 3.18.1)[12]. The edgeR model was constructed using a single pairwise comparison between two groups (Heat-shocked vs Non Heat-Shocked). The dispersion was estimated using the qCML method (estimateDisp). Differential expression between the two groups was performed using the quasi-likelihood (QL) method and a QL F-test (glmQLFit, glmQLFTest). Transcripts with a greater than background level of expression (mean log CPM > 0) and a low false discovery rate (FDR < 0.05) were used in the downstream analysis below.

Gene ontology, functional annotation, and KEGG annotation

To understand the biological significance of the genes displaying heat-induced expression, we investigated (i) gene ontology (GO) (*i.e.*, detailed annotations of gene function, related biological processes, and gene product cell locations) and (ii) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps (*i.e.*, annotations of gene metabolic and cellular functions). First, the transcripts were searched against NCBI's non-redundant (nr) protein database using BLASTX. The search was restricted to arthropods and employed an e-value cut-off of $10e^{-5}$. Transcripts were annotated with GO terms using BLAST2GO program[13] and an e-value cut-off of $10e^{-5}$. A second layer of GO terms was added to the transcripts using InterProScan online[14] and WEGO software[15] was used to functionally classify the terms. Second, the transcripts were annotated for biochemical pathways using the KEGG[16] Automatic Annotation Server (KAAS) for ortholog assignment and pathway mapping[17].

Results

Thermal tolerance and heat hardening

To evaluate heat-tolerance capabilities in *O. robustior,* survival assays of 6 groups made from 10 workers, each exposed to various thermal regimes for three hours, were performed (Fig.1). Survival was of 100% at 25°C and 42°C, then fell down proportionally with stress intensity to reach a value close to 0% after three hours at 49°C (mean percentage of survival ± s.d.: 1.66 ± 3.73%). We found no evidences of heat-hardening: pre-exposure of workers to the non-lethal temperature of 37°C for 2 hours, followed by exposure to 47°C for 3 hours, seemed to decrease survival (35.55 ± 22.67%) as compared to the non-pre-exposed workers (68.61 ± 16.63%; p = 0.055).

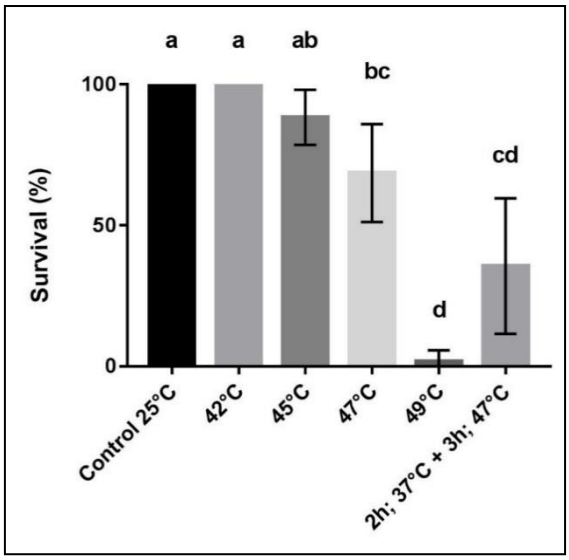

Figure 1: Mean ± s.d. survival rates of *O. robustior* workers after three hours of exposure to various heat-regimes. Exposure to 37, 42 or 45°C for 3h did not cause significant differences in mortality rates. Survival decreased dramatically after 3h at 49°C. A mild heat hardening of 2h at 37°C followed by heat stress of 3h at 47°C decreased survival. Different lowercase letters indicate significant differences between treatments (one-way ANOVA and Tukey *post hoc* test, p <0.05).

Constitutive and induced levels of Hsc70s

Western blot analyses of the expression levels of Hsc70s in *O. robustior* indicated no difference between control (3 hours, 25°C) and heat-shocked (3 hours, 45°C) workers (Fig.2A). The amount of Hsc70s detected was standardized on the histone H3 protein and a ratio of detection was calculated between heat-shocked workers (HS) and non-heat shocked workers (NHS). This ratio did not significantly differ from 1 (p value > 0.05; Fig.2B), indicating similar levels of Hsc70s accumulation under both conditions. As opposed, no Hsp70s was detected in *D. melanogaster* in control conditions, but the protein was heavily heat-induced in stress conditions.

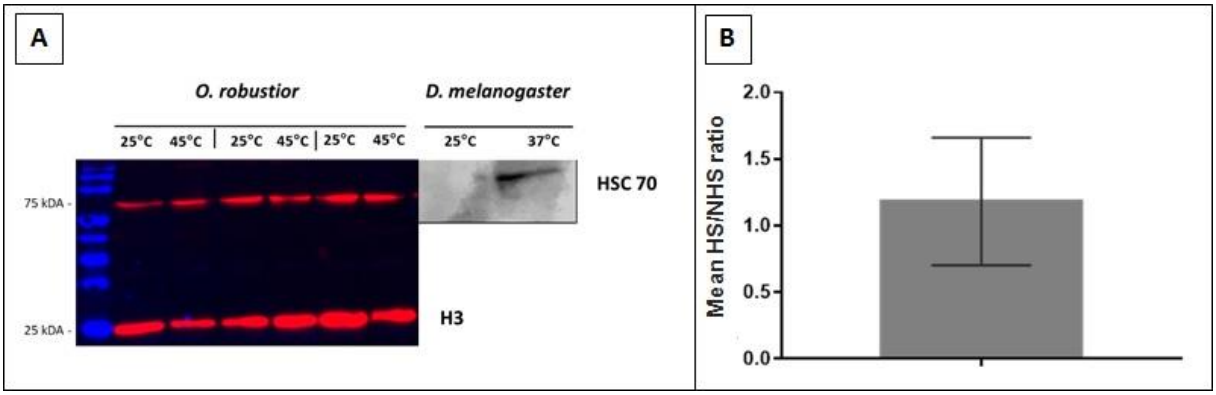

Figure 2.**A**. Fluorescent detection by western blotting of the presence of Hsc70s in control conditions (25°C) and heat-shock conditions (40°C) for *O. robustior* and *D. melanogaster* (n= 3 for each condition). *O. robustior* constitutively expressed strong levels of Hsc70s that are not further induced by heat-stress. As opposed, *D. melanogaster* does not constitutively express Hsp70s, but the proteins are heavily induced in stressful conditions. **B.** Mean ratio of standardized signal detection between heat-shocked (HS) and non-heat-shocked (NHS) *O. robustior* workers. The ratio does not significantly differ from 1.

Identification of heat-induced transcripts

We performed a differential gene expression analysis between 4 groups of control workers (4h, 25°C) and 4 groups of heat-stressed workers (4h; 45°C). A total of 18,975 transcripts (including isoforms) were identified. Of these, 450 displayed a significant regulation in response to heat stress (FDR < 0.05; FC > 2) and were qualified as differentially expressed sequences (DESs; Fig.3). Expression was downregulated for 107 DESs and upregulated for 343 DESs.

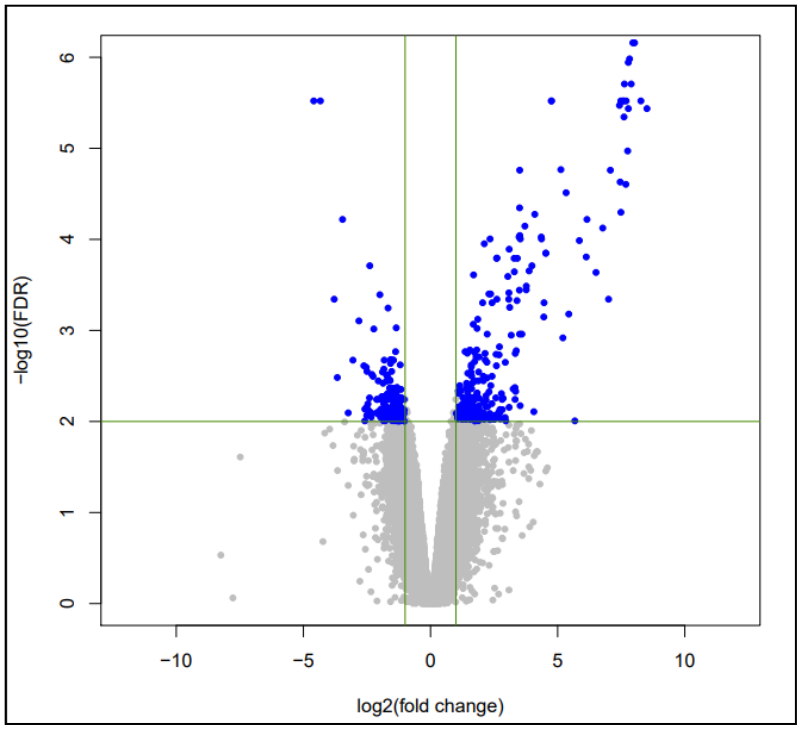

Figure 3. Relationship between fold change (FC) and false discovery rate (FDR) of the differentially expressed transcripts in response to heat-stress in the ant *O. robustior*. The 450 transcripts with above background levels of expression (FC > 2) and an FDR < 0.05 were selected for further screening (*i.e* transcripts shown above and outside green lines).

Similarity annotation

When the 450 DESs were queried against the NCBI nr protein database, 241 sequences (53%) with a high degree of homology were retrieved. Annotation was reliable, as most hits had e-values < $1e^{-180}$. The most represented taxa for the best hit of each match were other ant species, particularly those in the subfamily *Myrmicinae* such as *Atta* and *Acromyrmex*, a result that reflects *O. robustior*'s phylogenetic history.

Among those transcripts, the main functional class was linked to molecular chaperoning and co-chaperoning with 55 transcripts (23%; Fig.4). Other classes involved the regulation of the cell-cycle, autophagy and apoptosis (20; 8%), antioxidative mechanisms (20; 8%), protein degradation (14; 6%), lipid metabolism as well as phospholipid membranes composition (15; 6%), and low molecular weight molecules synthesis and transport (12; 5%). Partial results for relevant functional classes involved with heat-tolerance mechanisms are given in Fig.4

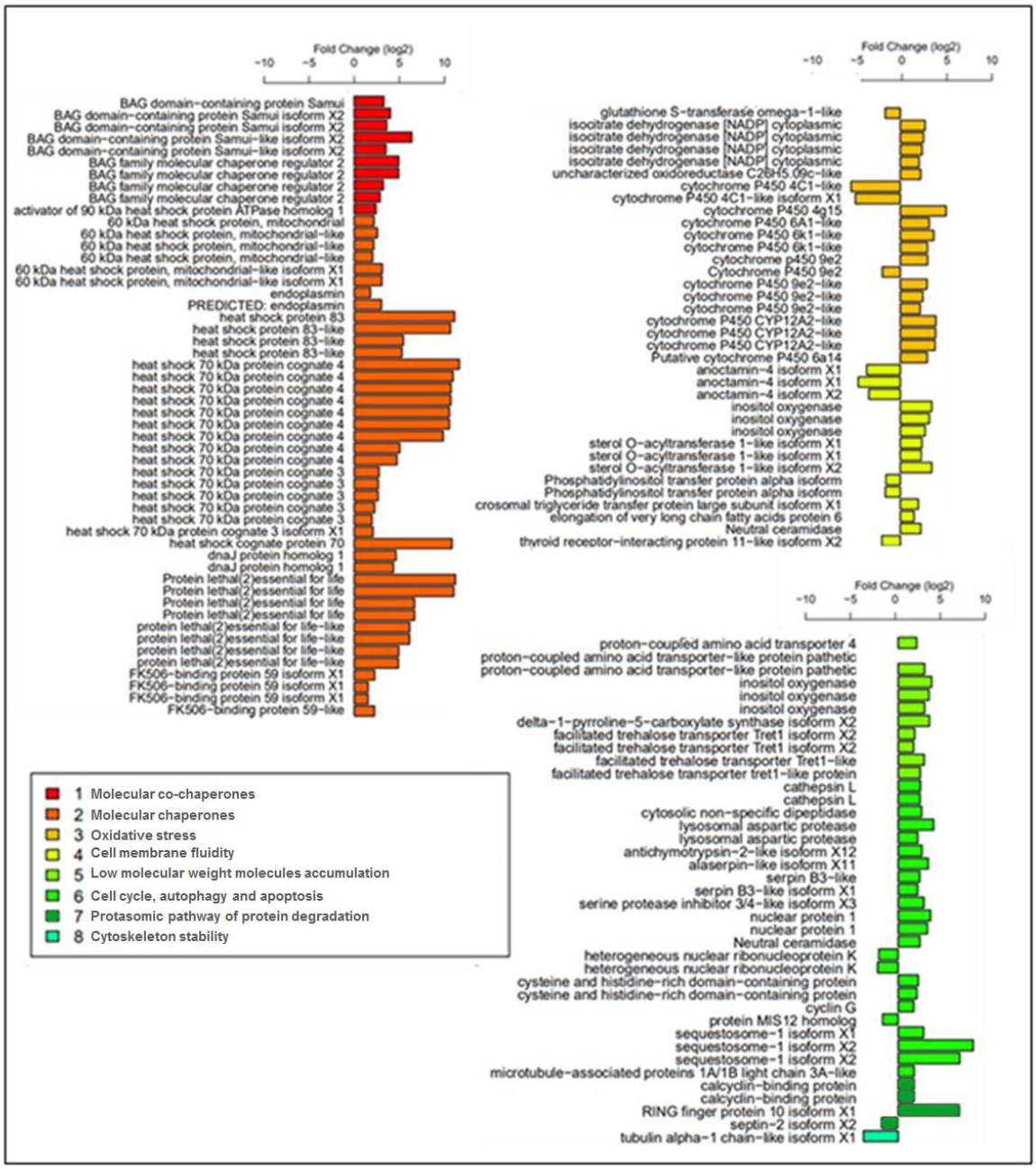

Figure 4. Functional classification of transcripts involved with molecular co-chaperoning (red), molecular chaperoning (orange), oxidative stress (light orange), cell-membrane fluidity (yellow), accumulation of low-molecular weight molecules (light green), cell-cycle regulation, apoptosis and autophagy (green), proteasomal degradation of proteins (dark green), and cytoskeleton stability (cyan).

Molecular chaperones involved in the heat-shock response

Of the 450 DESs, 55 were associated with either molecular chaperones or with co-chaperones involved in protein folding This indicates that these proteins represent a major part of the heat-shock response in *O. robustior*. We found members among the 5 major conserved families of Hsps (Table 1): 4 transcripts matched up with Hsp83 and 2 with Gp93, both belonging to the Hsp90 family. Fifteen transcripts matched up with Hsp70 proteins, 6 with the Hsp60 mitochondrial molecular chaperone and 8 with the protein Efl21l encoded by *lethal(2)essential for life (l(2)efl)*, which is a member of the Hsp20 family. Among molecular co-chaperones, two transcripts matched up with DnaJ homolog subfamily A member 1 (DnajA1) which belongs to the Hsp40 family, nine with the BCL-2-associated athanogene protein 2 (BAG-2), and one

transcript was associated with the activator of 90-kDa heat-shock protein ATPase homolog 1 (AHA1).

Comparison of the HSR under heat-stress between the Namib ant *O. robustior* and the Saharan ant *C. bombycina* showed that the transcripts associated with Hsc70-4, protein Efl21l, and BAG-2 are the most up-regulated in both species (> 10 FC; Fig.4). However, no transcript associated with Hsp100s or Hsc70-5 was identified to be heat-induced in *O. robustior* while they were in *C. bombycina*[10]. Furthermore, *O. robustior* induces more transcripts related to Hsp90s, Hsp60-mitochondrial, small heat-shock proteins and BAG2 regulators than the silver ant does.

Protein family	Protein	Number of associated transcripts in *O. robustior*	Number of associated transcripts in *C. bombycina*
Molecular chaperones			
Hsp100	ClpB	0	1
Hsp90	Hsp83	4	1
	Gp93	2	1
Hsp70	Hsc70-5	0	8
	Hsc70-4h	9	3
	Hsc70-3	6	1
Hsp60	Hsp60 mitochondrial	6	1
Hsp20	Efl21	8	2
Co-chaperones			
Hsp40	DnajA1	1	2
BAG proteins	BAG2	4	1
	Samui	5	0
AHA1	AHA1	1	1

Table 1. Summary of the molecular chaperones and folding co-chaperones found among the DESs in the ants *O. robustior* and *C. bombycina*, queried against the NCBI non-redundant protein database (arthropod records only) using BLASTX ($<10e^{-5}$).

KEGG annotation of the transcripts

For the 450 DESs tested, the main metabolic KEGG pathways found were associated with the isoprenoid backbone synthesis pathway, the arginine and proline metabolism and the pentose phosphate metabolism.

Discussion

Our results show that *O. robustior* is a species that is well-adapted to the peculiar conditions created by its thermal scavenging behavior in the Namib Desert. Workers are able to survive body temperatures of more than 45°C for several hours, express constitutively molecular chaperones associated with thermal tolerance[8], and regulates genes involved with molecular chaperoning and mitochondrial protection in response to heat-stress.

Interestingly, the physiological strategies for *O. robustior* workers to enhance their thermal tolerance are comparable to those found int the Sahara silver ant *C. bombycina*. Both species are able to tolerate elevated amounts of body temperatures for extended periods of time and constitutively express high levels of Hsc70 prior to heat-exposure, without displaying heat-hardening capabilities[9]. At the transcriptomic level, molecular chaperoning is a prevalent part of the heat-shock response in both species to stabilizes important cellular processes[10]. However, albeit these responses to heat-stress display striking similarities, they also differ on key elements that will be discussed hereafter.

First, when it comes to survival to heat-stress, it appears that *O. robustior* workers are able to tolerate slightly warmer temperatures than workers of *C. bombycina*, for longer periods of time. After 3 hours exposure to 45°C, survival rate in *C. bombycina* falls to 40 ± 10%[9], whereas it reaches 85 ± 10% for *O. robustior* workers. This contrasts with the recorded higher CT_{max} for *C. bombycina* (53.6°C)[1] than for *O. robustior* (51.2°C)[4]. However, it is to be noted that measurements of CT_{max} are actually poorly reproducible and quite dependent on the methodological context. CT_{max} thus needs to be considered only as an estimation of upper thermal-tolerance limits[18,19]. In any case, this difference likely reflects a better adaptation of *O. robustior* workers to tolerate body temperatures. This is somewhat surprising, since summer day ground and air temperatures in the Sahara and in the Namib, desert are comparable, and foragers of both species are exposed to similar conditions while foraging. One potential explanation is that *O. robustior* workers truly experience warmer body temperatures than the silver ant workers do. In line with this, it has been shown that the silver coat of *C. bombycina* shifts down the thermal equilibrium of individuals of a few degrees by reflecting visible wavelengths through total internal reflection, an adaptation that is absent in *Ocymyrmex*[20,21]. Thus *O. robustior* might have evolved to comparatively tolerate slightly warmer thermal stresses from a physiological standpoint, a level of adaptation that could not have been selected in the silver ants because it is kept cooler by its unique morphological feature.

Third, detailed analysis of *O. robustior* molecular chaperones upregulated during heat-stress revealed divergent response strategies as compared with *C. bombycina* (Table 1). A first major difference stems from the fact that ClpB was not found to be heat-induced in *O. robustior*, while it is upregulated in the silver ant. ClpB forms a complex with Hsp70/40 proteins that resolves and solubilizes denatured protein aggregates[22]. Furthermore, *O. robustior* express many more BAG related transcripts than the silver ant does. BAG-domain containing protein assist Hsp70s activity in protein folding as well as regulating degradation through the ubiquitin-proteasome system[23,24]. This absence of ClpB induction correlated with the abundance of heat-induced BAG proteins could reflect a greater investment of *O. robustior* into protein turnover (*i.e*, recycling damaged proteins with *de novo* protein synthesis to replace them). In contrast, the induction of ClpB and reduced numbers of BAG proteins found in the silver ant might be consistent with a greater investment into proteome stability (*i.e* preserving and refolding denatured proteins with more limited protein synthesis). Cells proteostasis depends on the balance between protein folding and protein degradation, both mechanisms being complementary to maintain a proper amount of functional proteins. Our data suggest that the two species may favor different angles of the cellular protein quality control to achieve proteostasis.

A second major divergence between the Namib ant and the Sahara ant stems from the differential induction of mitochondrial-related molecular chaperones. No *hsc70-5* transcripts were found to be heat-induced in *O. robustior*, while they are the most numerous among molecular chaperones to be found in the silver ant (Table 1). By contrast, *O. robustior* express more transcripts related to *hsp60-mitochondrial* than the silver ant does. Thus, protecting mitochondria from heat-induced damages seems to be an important aspect of the HSR in both species. However, the molecular mechanisms involved in this process seem those two species of thermal scavenging ants.

Fourth, KEGG metabolic analysis of the 450 DEEs of *O. robustior* pinpointed heat-induction of enzymes linked with terpenoid backbone synthesis, proline metabolism and pentose phosphate pathway. So far, plants model-based experiments have provided evidence that terpenoid compounds and proline accumulation are related to protection against oxidative stress, especially against lipid peroxidation[25,26]. It could also be that terpenoid are involved in the ant's cholesterol synthesis pathways, which has a potential involvement in the adaptation of cell membranes fluidity to heat-stress[27]. Up-regulation of transcripts related to terpenoid and proline transport or biosynthesis in thermal scavenging ants naturally raises interest to their potential relevance in insects and would deserve further confirmation. The induction of pentose phosphate pathways in response to oxidative damages, on the other hand, has been documented in many organisms ranging from yeast to humans[28,29]. It has been proposed that such metabolic activity serves as maximizing NADPH output, which itself is an important co-factor of the glutathione-reductase enzyme whose activity maintains proper amount of reduced glutathione as a buffer against oxidative damages[29]. Interestingly, induction of the pentose phosphate pathway was also observed in heat-stressed *C. bombycina* workers through KEGG analysis[10]. This suggests that protecting cells against oxidative damages is another important aspect of the thermal scavenging response to heat-stress. The metabolic demands of running at elevated speed and under extreme heat-pressure is likely high, and such activity must generate large amounts of intracellular ROS whose potential toxic effects need to actively be kept in line.

Finally, numerous genes involved with cellular processes related to heat-tolerance were found to be differentially expressed in *O. robustior*: 20 transcripts were linked to protein and enzymes involved with regulating cell membrane fluidity, 21 transcripts were directly involved with regulating intracellular oxidative damages, and 20 more were linked to the regulation of the cell-cycle, autophagy and apoptosis. The detailed interpretation of heat-shock effects on those molecular pathways, as well as their comparison with those of *C. bombycina*, couldn't be performed so far. However, it is to be expected that from this comparison, more distinct and yet functionally convergent mechanisms supporting heat-tolerance between those two species could be identified.

In conclusion, our data indicate that parallel strategies aimed an enhancing body-tolerance of workers have evolved in two distantly related thermal scavenging ants. Functional convergent evolution thus seems to occur at the molecular level as a response the evolutionary pressures enforced by this unique desert behavior. Further molecular studies on other thermal scavenging ants will allow us, in time, to depict a broader picture on how evolution shaped cell-tolerance to heat-stress in organisms repeatedly exposed to short bursts of intense heat-stress.

References

1. Wehner, R., Marsh, A. C., & Wehner, S. (1992). Desert ants on a thermal tightrope. *Nature*, *357*(6379), 586.
2. Muser, B., Sommer, S., Wolf, H., & Wehner, R. (2005). Foraging ecology of the thermophilic Australian desert ant, Melophorus bagoti. *Australian Journal of Zoology*, *53*(5), 301-311.
3. Wehner, R., & Wehner, S. (2011). Parallel evolution of thermophilia: daily and seasonal foraging patterns of heat-adapted desert ants: Cataglyphis and Ocymyrmex species. *Physiological entomology*, *36*(3), 271-281.
4. Marsh, A. C. (1985). Thermal responses and temperature tolerance in a diurnal desert ant, Ocymyrmex barbiger. *Physiological zoology*, *58*(6), 629-636Christian, K. A., & Morton, S. R. (1992).
5. Extreme thermophilia in a central Australian ant, Melophorus bagoti. *Physiological Zoology*, *65*(5), 885-905.
6. .Moreau, C. S., Bell, C. D., Vila, R., Archibald, S. B., & Pierce, N. E. (2006). Phylogeny of the ants: diversification in the age of angiosperms. *Science*, *312*(5770), 101-104.
7. Marsh, A. C. (1985). *Aspects of the ecology of Namib Desert ants* (Doctoral dissertation, University of Cape Town).
8. Gehring, W. J., & Wehner, R. (1995). Heat shock protein synthesis and thermotolerance in Cataglyphis, an ant from the Sahara desert. *Proceedings of the National Academy of Sciences*, *92*(7), 2994-2998.
9. Willot, Q., Gueydan, C., & Aron, S. (2017). Proteome stability, heat hardening, and heat-shock protein expression profiles in Cataglyphis desert ants. *Journal of Experimental Biology*, jeb-154161.
10. Willot, Q., Mardulyn, P., Defrance, M., Gueydan, C., & Aron, S. (2018). Molecular chaperoning helps safeguarding mitochondrial integrity and motor functions in the Sahara silver ant Cataglyphis bombycina. *Scientific reports*, *8*(1), 9220.
11. Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC bioinformatics*, *12*(1), 323.
12. Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139-140.
13. Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, *21*(18), 3674-3676.
14. Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C., ... & Pesseat, S. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics*, *30*(9), 1236-1240.
15. Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., ... & Wang, J. (2006). WEGO: a web tool for plotting GO annotations. *Nucleic acids research*, *34*(suppl_2), W293-W297.
16. Kanehisa, M. & Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
17. Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A. & Kanehisa, M. KAAS: an automatic genome annotation and pathway reconstruction server. *Nucleic Acids Res.* **35**, W182–W185 (2007).
18. Terblanche, J. S., Deere, J. A., Clusella-Trullas, S., Janion, C., & Chown, S. L. (2007). Critical thermal limits depend on methodological context. *Proceedings of the Royal Society of London B: Biological Sciences*, *274*(1628), 2935-2943.
19. Hoffmann, A. A., Chown, S. L., & Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they?. *Functional Ecology*, *27*(4), 934-949.
20. Shi, N. N., Tsai, C. C., Camino, F., Bernard, G. D., Yu, N., & Wehner, R. (2015). Keeping cool: enhanced optical reflection and heat dissipation in silver ants. *Science*, aab3564.
21. Willot, Q., Simonis, P., Vigneron, J. P., & Aron, S. (2016). Total internal reflection accounts for the bright color of the Saharan silver ant. *PloS one*, *11*(4), e0152325.
22. Doyle, S. & Wickner, S. Hsp104 and ClpB: protein disaggregating machines. *Trends Biochem. Sci.* **34**, 40–48 (2009).
23. Lüders, J., Demand, J., & Höhfeld, J. (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *Journal of Biological Chemistry*, *275*(7), 4613-4617.
24. Qin, L., Guo, J., Zheng, Q., & Zhang, H. (2016). BAG2 structure, function and involvement in disease. *Cellular & molecular biology letters*, *21*(1), 18.
25. Grassmann, J., Hippeli, S., & Elstner, E. F. (2002). Plant's defence and its benefits for animals and medicine: role of phenolics and terpenoids in avoiding oxygen stress. *Plant Physiology and Biochemistry*, *40*(6-8), 471-478.

26. Liang, X., Zhang, L., Natarajan, S. K., & Becker, D. F. (2013). Proline mechanisms of stress survival. *Antioxidants & redox signaling*, *19*(9), 998-1011.
27. Pierre Boiteau, B. Pasich et Albert Rakoto Ratsimamanga, Les Triterpénoïdes en physiologie végétale et animale, Paris, Éditions Gauthier-Villars, 1964
28. Slekar, K. H., Kosman, D. J., & Culotta, V. C. (1996). The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection. *Journal of Biological Chemistry*, *271*(46), 28831-28836.
29. Kuehne, A., Emmert, H., Soehle, J., Winnefeld, M., Fischer, F., Wenck, H., ... & Zamboni, N. (2015). Acute activation of oxidative pentose phosphate pathway as first-line response to oxidative stress in human skin cells. *Molecular cell*, *59*(3), 359-371.

Discussion

The work undertaken during this thesis has been focusing on heat-tolerance strategies in thermal scavenging ants. The discovery of total internal reflection as an optical mechanism for thermoregulation (Chapter I)[1] as well as the functional characterization of the molecular chaperones involved in those ants' heat-shock response (Chapter II, III)[2,3] pushes forward our still fragmental knowledge of heat-tolerance mechanisms in desert-adapted species. Second, it lays the foundations for further works, especially in the field of ecophysiology, to infer broader evolutionary mechanisms of heat-tolerance in ecthotherms (Chapter IV). Ultimately, the objective of the presented research is to provide an understanding of the different layers of adaptations that interlinks together as a response to harsh environmental conditions.

A lifeform's adaptation to its thermal environment results from back and forth exchanges between pressures and responses and can thus be seen as a dynamic equilibrium. To understand this equilibrium is to understand the relative contribution of all it's factors, and how they integrate within each other. A similar result (*i.e* a fitted response to a given microclimatic conditions) can be achieved in several ways, depending on the evolutive emphasis put on behavioral, morphological or physiological features. Thus, while it is important to grasp the full measure of adaptive equilibriums as a global response, the practical investigations of this thesis had to be restricted only to some specific aspects of morphology (Chapter I) and physiology (Chapter II, III and IV). Here, we will discuss how those aspects can equilibrate with each other and with their microclimatic pressures, in the light of our own results and in a global context in ectotherms.

Morphological adaptations to thermal stress in ants

First, when it comes to morphology, surprisingly few works have focused on its relation to thermal tolerance in thermal scavenging ants. Most centered on leg morphology as well as worker polymorphism, and their potential adaptive to thermal stress[4,5,6]. Thermal scavenging ants all have elongated limb segments that allow them to elevate their body away from the burning ground while reaching considerable running speeds[4]. This feature is shared with other ectotherms having to run on hot surfaces, as is the case for some diurnal beetles from the Namib and the Negev deserts (Fig.9)[7,8].

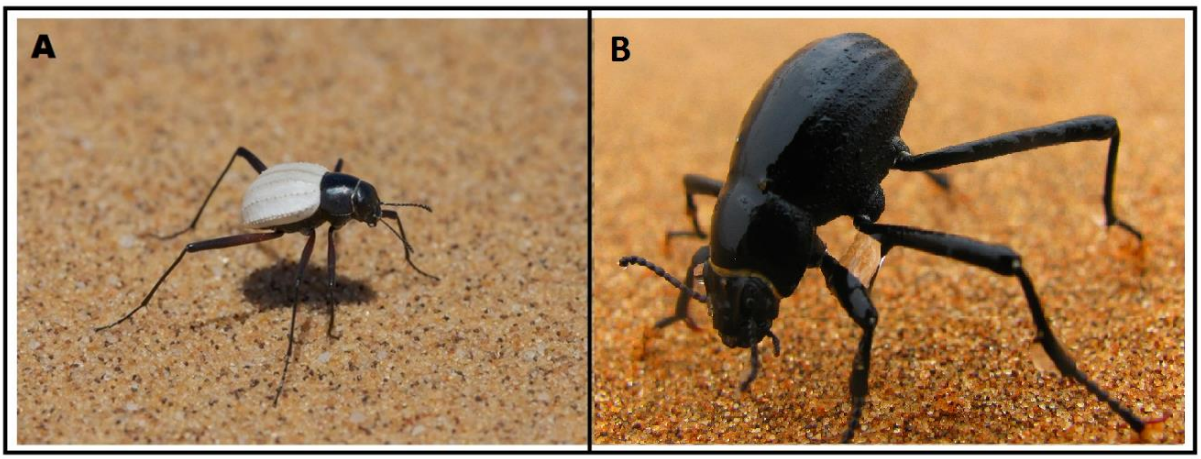

Figure 9. Namib beetles from the genus *Onymacris* display, similar to thermal scavenging ants, very long legs and high running speed of up to 90 cm/sec (**A.** *O bicolor*, **B.** *O. unguicularis*). They are well adapted to locomotion on burning surfaces and are among the only active ectotherms during the hottest part of the day in the Namib, alongside with *Ocymyrmex* species[7]. ©Piazzi. M (A), Anderson. J (B).

Larger ectotherms are thought to be at advantage over smaller ones for they have superior thermal inertia, hence reach thermal equilibrium with their surrounding over a longer period of time [9]. Considering polymorphic thermal scavenging ant species like *Cataglyphis* and *Melophorus*, larger individuals could be better adapted to withstand short bursts of intense heat-stresses, and this was to some extent shown in *Cataglyphis velox* and *Cataglyphis cursor*[5,6]. Thermal inertia alone is likely accountable for such observation (as big workers won't heat as fast as smaller ones), and this could indeed give an edge to larger workers when it comes to foraging further away from the nest. However, large workers with slower heat-dissipation are also at disadvantage in use of thermal refuges (when workers rest in the shade or climb on stems to cool themselves). Being larger can also become impairing when navigating in some terrain, such as sand dunes (smaller workers of *C. bombycina* are much more agile in climbing sandy slopes than bigger individuals, *personal observation*). Finally, polymorphism is widespread across the ant family regardless of any thermal environmen specificities. In the case of thermal scavenging ants, *Cataglyphis* and *Melophorus* species mostly are polymorphic, while *Ocymyrmex* are not. Thus, the potential contribution of size to thermal tolerance in ants is somewhat unclear to this day, which likely reflects that size can quickly become a context-dependent restrictive factor. Being big or polymorphic is thus not an obvious evolutionary trend in thermal scavenging ants (nor is it in other ectotherms adapted to high ground temperatures), as opposed to be long-legged.

Second, another factor joining both behavior and morphology that has been of interest to understand heat-hardiness in *Cataglyphis* is the ability of some species to run with the gaster pointing up (Fig. 10A). It has been hypothesized that elevation of vital organs further away from the burning ground was a behavior deployed to survive harsh thermal conditions outside the nest. Indeed, a 4.4°C difference in gaster temperatures was observed between same-sized workers of species running with or without this gaster up position[10]. However, a later study has linked this ability with an evolutionary advantage in locomotion rather than a mechanism of heat-tolerance, a raised gaster requiring less torque energy to take tight turns at high running speed[11]. In addition, this ability is restricted to some groups of *Cataglyphis* only (and some rare species of non-thermal scavenging ants, especially inside the *Camponotus* genus), while some of the most thermophilic species such *C. bombycina* are unable to do so. This behavior is further absent from the *Ocymyrmex* and *Melophorus* genera. Thus, so far, we lack systematic evidences linking the ability to run with the gaster pointed up with thermoprotection. While it cannot be excluded that it could indee be a context-depend thermoprotective factor for some *Cataglyphis*, it seems likely that such ability is primarily linked with an advantage for fast navigation, especially when engaging into erratic trajectories.

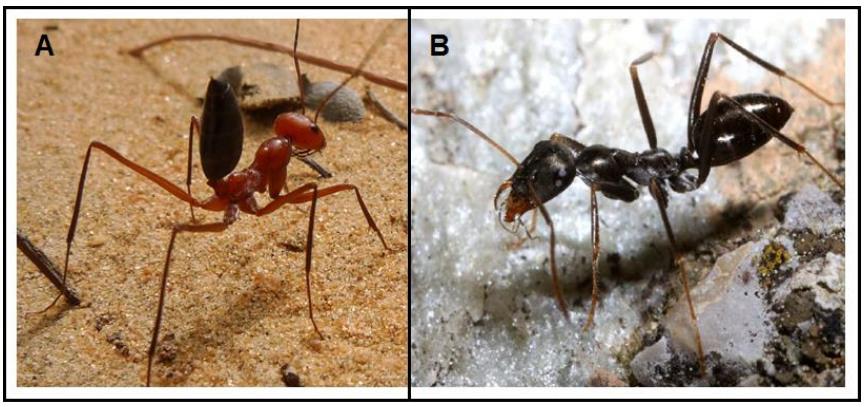

Figure 10. A. A minority of *Cataglyphis* species has the ability to engage into foraging activities with the gaster raised up, such as species from the group bicolor. **B.** The majority of *Cataglyphis* species, as opposed, engage into foraging activity with the gaster laid flat, such as species from the group cursor. ©Cerda. X (A), Lebas. C (B).

Third, an essential element of the thermal budget of ectotherms is coloration[9]. Solar radiation at sea level is roughly made of 3% ultraviolet, 40% visible and 57% infrared wavelengths[12]. Simply put, that means that white surfaces reflect 40% of the sun's energy while black surfaces absorb it. Thus, under similar radiative conditions, black ectotherms are believed to heat faster and reach higher thermal equilibriums than lighter-colored ones. From there stems the *thermal melanism hypothesis*[12,13]. This hypothesis posits that dark coloration is beneficial for ectotherms under conditions of low ambient temperatures because it results in faster heating rates and higher body temperatures. So far, this hypothesis has found ample support, for it seems that a positive relationship between darker color and lower mean annual radiation exists in terrestrial ectotherms[14,15]. Taken backward, this hypothesis also states that insects would logically need to protect themselves against overheating by means of lighter color in areas with higher mean temperatures. This trend also seems to confirm in the light of climate change, for selection of lighter colorations in European insects does seem to correlate with elevation of mean annual temperatures[16]. The thermal melanism hypothesis would therefore logically predict that thermal scavenging ants would be advantaged by lighter colorations. Yet, this is not the case, for *Cataglyphis* and *Ocymyrmex* species mostly are of dark cuticle color[17]. This could be explained by taking the potential need for UV protection into account. Thermal scavenging ant genera possess a cuticle only a few micrometers thick[18]. Protection from UV radiative damages likely sets another tradeoff for darker skin color in the middle of the desert even at the expense of increased heating rates. This is especially true if foragers venture far away from the nest for longer periods of time. Amazingly, one species of *Cataglyphis* has developed a unique way around this conundrum: the Saharan silver ant *C. bombycina*. Our work shows that workers are covered with a dense array of prismatic hairs that allows ten times more visible light to be reflected through total internal reflection, which gives the ant its color (Chapter I). In addition, in the mid-infrared range of the spectrum where the solar energy is negligible, they are thermally transparent and thus enable to radiate off excess heat even under sunny conditions[19]. Both effects provide the silver ants hairs with remarkable thermoregulatory properties, enabling them to maintain a lower thermal steady state. As opposed to all others Saharan *Cataglyphis* species, the silver ant harness both the abilities to be protected against UV damages and to reflect visible wavelengths to lower its inner thermal equilibrium for a few degrees. The use of total internal reflection to thermoregulate is, to our knowledge, unique in the animal kingdom and novel morphological adaptation to cope with harsh thermal constrains.

Fourth, it has been argued that in some cases, color might not even be relevant for ectotherm's thermal budget, as depicted by the black namib beetle paradox[20]. Again, in opposition to the thermal melanism hypothesis, the white species beetle of the genus *Onymacris* (*O. bicolor*; Fig.9A) seems to be at no particular advantage regarding thermal budget as compared to its black relative (*O. unguicularis*, Fig.9B)[21]. Because of the beetle size and superior thermal-inertia (as compared to ants), it's running speed, it's activity during high wind velocity and its use of thermal refuges, it has been argued that coloration doesn't play a significant role in the heat-equilibrium of the animal[21]. This actually highlights how hazardous it is to extrapolate global values of adaptive traits to heat tolerance mechanisms in ectotherms. That difficulty arises because some features (such as size or activity during high winds) might compensate for others (such as color) in a species-dependent context and still results in individuals fitted to survive similar conditions.

Our studies reveal another example of how adaptive features can compensate for each other in body-heat survival. Comparison between *C. bombycina* and *O. robustior* (Chapter IV) shows that *O. robustior* seems to have invested more into the physiological aspects of heat tolerance, thus being able to survive slightly warmer temperatures for longer periods of time than *C. bombycina* does. However, the silver ant is effectively protected against solar radiations by its silver coating, and both species are able of foraging efficiently in similar microclimatic

conditions. It could well be that in that case, a morphological protective feature (hairs) makes greater investments in physiological heat-tolerance mechanisms useless, and vice versa. Another example would be that some bigger, polymorphic species of *Cataglyphis* tend to have lower physiological heat tolerance thresholds but divide foraging activity according to worker size, larger workers being able to sustain elevated temperatures for more time due to their superior speed and thermal inertia. As opposed, it seems that smaller and monomorphic species of *Cataglyphis* tend to invest more into physiological pathways of heat-tolerance, thus being able to forage at similar temperatures than bigger workers of polymorphic species do[10]. Thus, tolerance adaptation to heat is not a one-way road but can take multiples turns integrating various aspects and tradeoffs between of behavior, morphology and physiology. This produce finely tuned response that rarely is identical, even between relatives exposed to similar conditions.

The relative contribution of the heat-shock response to thermal tolerance

A very well-supported idea is that the mechanisms of heat-shock response are plastic from an evolutionary perspective[22-24]. This can be attributed to the fact that eukaryotic organisms regulate their HSR at many complimentary levels, multiplying angles for evolutionary pressure to create variant and peculiarities in this system. For example, the activation temperature of the transcription factor HSF1 (which is the main transcription factor responsible for activating the HSR genes upon heat-shock, by binding to heat-shock elements in promoter region of target genes)[23] may greatly vary between closely related species and in response to acclimation, with consequences that the triggering of the HSR will occur at different temperatures[23-27]. Heat-shock elements (HSE) themselves are highly mobile and can confer differential susceptibility of genes to the HSF1 induction pathway[1,28,29]. Furthermore, high levels of functional redundancies exist among heat-shock proteins and their co-factors, allowing their differential evolution and functionalization across taxa[28-34]. For example, it has been described in most organisms from *Drosophila* to mammals that Hsp70 was a molecular chaperone induced in response to stress only. As opposed, its related cognate Hsc70 was not head-inducible but had a rather low constitutive expression, fulfilling a housekeeping function in protein synthesis folding (both proteins are, in human, 86% identical)[35]. However, Hsp70 has been lost in hymenopterans. Instead, the functional role of Hsp70 seems to have been taken up by Hsc70, which further duplicated[26]. Accordingly, in ants, *hsc70-4 h1* and *hsc70-4 h2* are among the most overexpressed genes in response to heat-stress[2,28] and are two orthologs of the non heat-inducible housekeeping *Drosophila hsc70* gene. A final layer of regulation can further be added by the relative contribution of other processes, such as cellular epigenetic control[36,37.] Taken together, this hub of regulatory mechanisms can create a complex but also species-specific heat-shock response as an answer to detrimental conditions.

Some of the potential variability in the mechanisms of Hsp-mediated stress tolerance are highlighted in this thesis (Chapter II, III and IV). By comparing expression patterns of some *Cataglyphis hsps* genes with other non-thermal scavenging ants (such as the wood and *Aphaenogaster rudis* and the harvester ants *Pognonomyrmex barbatus*)[28], we showed specific heat-induction of *hsc70-5, hsp60-m* and genes related to sHsps[2]. This was confirmed by further transcriptomic analyses, allowing us to infer that some specific means of protecting mitochondria and motor functions exist in *Cataglyphis* and are absent in other ants[3]. And last, by comparing two distantly related genera of thermal scavenging ants, we unraveled both similarities and differences in identity and expression patterns of some molecular chaperones involved in the HSR. Similar to *Cataglyphis*, *Ocymyrmex* does up-regulate *hsp60*-mitochondrial and *sHsps* genes in response to heat-stress, a thing that hasn't been documented in non-thermal scavenging ants so far. As opposed to *Cataglyphis* though, *Ocymyrmex* seems

to have lost the ability to up-regulate *ClpB* (implied in re-solubilizing denatured protein aggregates)[36] and *Hsc70-5* genes (that is mitochondrial). Ath the functional level though, *O. robustior* could very well make up for it through a more diversified used of BAX co-chaperones involved in protein turnover, and a more diversified use of hsp60-mitochondrial proteins. As argued previously, it highlights that some physiological and molecular adaptations could compensate for others but still converge to confer protection to the same functional traits. Ultimately, this can result in an adequate adaptation of organisms to similar constrains (Chapter IV).

As one of the few general rules that could be extrapolated on how life adapts to climatic conditions, evidences support that organisms living in very stable thermal environment do not in general respond to heat stress with an inducible HSR, whereas organisms that occupy highly variable environments induce the HSR frequently, with the subsequent ability to mount a heat-hardening response[22]. Species ability to up-regulate HSR genes is thus constrained by their evolutionary history and reflects their own thermal preferences. This rule can verify inside different ectotherm taxa[40-42], and even verify through the different life stages of a single organism. Larvae of the flightless midge *Belgica* antarctica spend most of their life in a thermally buffered soil medium alongside the coast of the Antarctic Peninsula. Those larval stages express very high level of constitutive Hsp70s without any capability of mounting a heat-hardening response[42]. As opposed, adults found crawling on the surface and that are exposed to more variable thermal conditions up-regulate *hsps* in response to heat stress and concurrently are capable of generating thermoprotection. This differential inducibility of Hsps during a single organism's life stage underpins how evolutionary plastic this response can be to answer thermal pressures.

Chapter II underlines how this variability is of importance to understand HSR response in *Cataglyphis* ants. *C. bombycina* seeks for food in a narrow range of elevated temperatures (46.5– 53.6°C) no matter the time of the year[43]. They have evolved to deal with always stressful conditions outside their nests. It is thus advantageous for *C. bombycina* workers to express high constitutive levels of Hsc70s, but they have little need for a finely tunable response and seem to lack the ability to mount an efficient heat-hardening[2]. As opposed, *C. mauritanica* is exposed to on average lower foraging temperatures in the Atlas Mountains but can expect to meet a greater range of thermal conditions when foraging. Thus, it is advantageous for *C. mauritanica* workers not the spend too much energy into constantly synthetizing elevated amount of Hsc70s, but they have greater needs for a finely tunable system. Accordingly, *C. mauritanica* workers show more up-regulation of HSPs related genes than *C. bombycina* when exposed to heat-stress, display heat-hardening, and when making use of that ability are even able to survive harsher treatment than the silver ant does[2]. Those species belong to the same genus, but thermal pressures have driven differential evolution of their HSR as a response to their ecological constraints. This might stem from differences in the structure and sensibility of HSF1, mobility and rearrangements of HSEs in promoter regions of HSPs genes, or even duplications or losses of function of certain HSPs. Further work would be needed to elucidate the molecular mechanisms behind this rapid speciation of the HSR in *Cataglyphis*. However, what remains is that their HSR peculiarities, as is the case for most organisms, seems to directly reflect the microclimatic conditions those ants are exposed to. Acquiring a deep knowledge of a model's microclimatic conditions is thus critical to put ecophysiological data into a comprehensive evolutionary context.

A final word should be made concerning the practical limitation of this work. We've been using stress treatments of 40 to 45°C for 3 hours on workers. Such treatments were not intended to mimic natural heat-stress that thermal scavenging ants encounter in the wild, for workers usually perform foraging trips of around 30 minutes and use thermal respites regularly to cool off. Thus, our experimental conditions were somewhat harsher than conditions those insects are

generally be exposed to in their habitat. An integrative approach would have been to perform experiments on ranging temperatures as well as ranging times of exposure, but that would have multiplied considerably the need for biological material. Practical limitation of ants' availability prevented us to follow workers' responses in a time and temperature dependent manner. It was therefore a deliberate decision to make use of treatments that would force individuals to make use of the full extent of their physiological thermal-tolerance mechanisms. We chose to extract data from stress temperatures already inducing significant mortality. It is likely that in those conditions, transcription of some genes involved with shorter responses to milder heat-stress was already off and thus went undetected. It is also likely that some of the transcripts detected, notably linked with cellular death, were extracted from noncritical tissues that were already beyond recovery in some workers and thus not involved with heat-tolerance mechanisms (we made certain that workers surviving experimental treatments would recover in the next few hours/days at a 100% rate). Thus, while our approach had inherent limitations, we optimized experimental procedures and interpreted our results accordingly to yield relevant, albeit incomplete, data.

Perspectives

The thermic constraints put upon thermal scavenging ants are among the harshest known to land arthropods. Such a challenge led to convergent evolution of behavioral, morphological and physiological features to cope with short episodes of intense of heat-stresses. While this work, alongside many others, has made a leap forward the understanding of their adaptation to heat-stress, this is only but an incomplete picture. However, our evolutionary vision is limited, for we lack a comprehensive overview of heat-stress tolerance mechanisms in organisms exposed to transient bursts of intense heat-stresses, as well as with social insects in general. Thus, by analyzing in a comparative phylogenetic framework *Cataglyphis*, *Ocymyrmex*, and *Melophorus* species, many interesting questions could still be addressed at this point.

For example, from a morphological perspective, their respiratory system could unveil novel mechanisms for enhanced oxygen diffusion under high thermal pressures, or to prevent water loss. Thermal imaging could also be used to estimate precisely workers thermal equilibrium during foraging trips. Finally, investigating the relationship between the melanisation of the cuticle and the need for UV protection in thermal scavenging ants could be another lead to understand the seemingly contradiction between their darker colors, and the thermal melanism hypothesis.

In a physiological context, we have no understanding of how those insects specifically regulate their cell membrane fluidity while supporting elevated body temperatures. Phospholipid and cholesterol biosynthesis, as well as pathways modifying saturation of fatty acid tails, are thus of particular interest in those taxa. Our knowledge of how those insects regulate their metabolism, how they cope with oxidative stress, as well as the relative contribution of their autophagic pathways to heat-stress survival is also virtually nonexistent. To this regard, it would be especially interesting to explore the formation mechanisms of stress-granules involved in the protection of mRNA during heat-shock. Thermal scavenging ants could also accumulate small molecular weight molecules with beneficial effects on protein stability and buffering oxidative damages. Finally, at the molecular level, it is to be expected that the very conformation of some proteins has been selected to confer enhanced resistance to heat-induced denaturation. From there could be gathered novel protein structures, and information about their potential role in the cellular homeostasis.

Another line of perspective would be to explore the molecular mechanisms underlying the fast speciation of the HSR in thermal scavenging ants. Using ATAC sequencing, we could characterize accessible chromatin regions, both outside and under heat-stress, which might partially shed some light to the mechanisms of constitutive Hsps expression observed in some

species. Using Chip-sequencing, we could characterize the interaction of transcription factors with DNA under heat-stress genome-wide, to unravel divergences in terms of mechanisms of *hsps* transcription during heat-stress. Finally, using whole genome sequencing, we could quantify and characterize HSE in promoter regions of *hsps* across species, as it was done for some other non-thermal scavenging ants[26]. Together, this integrative picture of what areas of the genome are accessible to the transcription machinery under heat-stress, what transcription factors actively play a role in thermal-tolerance mechanisms, and their relation to the HSE in promoter regions of *hsps* might yield novel knowledge on the evolutionary mechanisms behind the incredible heat-hardiness of those insects.

Thus, thermal scavenging ants could be fertile ground to understand how evolution can shape thermal tolerance and resistance in desert organisms from a cellular perspective. At the upper level the questions addressed in this thesis, and pieces of answers that were brought to them, integrate into an insightful picture of life's evolution in response to abiotic pressure. Each organism exposed to similar conditions will reach a fitted adaptive response whose underlying basis might change in reflection to their respective evolutive histories. This emphasis the need to interpret the value of adaptive traits a global context, but also beautifully illustrates the ingenuity and plasticity of life on Earth.

References

1. Willot, Q., Simonis, P., Vigneron, J. P., & Aron, S. (2016). Total internal reflection accounts for the bright color of the Saharan silver ant. *PloS one*, *11*(4), e0152325.
2. Willot, Q., Gueydan, C., & Aron, S. (2017). Proteome stability, heat hardening, and heat-shock protein expression profiles in Cataglyphis desert ants. *Journal of Experimental Biology*, jeb-154161.
3. Willot, Q., Mardulyn, P., Defrance, M., Gueydan, C., & Aron, S. (2018). Molecular chaperoning helps safeguarding mitochondrial integrity and motor functions in the Sahara silver ant Cataglyphis bombycina. *Scientific reports*, *8*(1), 9220.
4. Sommer, S., & Wehner, R. (2012). Leg allometry in ants: extreme long-leggedness in thermophilic species. *Arthropod structure & development*, *41*(1), 71-77.
5. Cerdá, X., & Retana, J. (1997). Links between worker polymorphism and thermal biology in a thermophilic ant species. *Oikos*, 467-474.
6. Clémencet, J., Cournault, L., Odent, A., & Doums, C. (2010). Worker thermal tolerance in the thermophilic ant Cataglyphis cursor (Hymenoptera, Formicidae). *Insectes Sociaux*, *57*(1), 11-15.
7. Nicolson, S. W., Bartholomew, G. A., & Seely, M. K. (1984). Ecological correlates of locomotion speed, morphometries and body temperature in three Namib Desert tenebrionid beetles. *African Zoology*, *19*(3), 131-134.
8. Krasnov, B., Ward, D., & Shenbrot, G. (1996). Body size and leg length variation in several species of darkling beetles (Coleoptera: Tenebrionidae) along a rainfall and altitudinal gradient in the Negev Desert (Israel). *Journal of Arid Environments*, *34*(4), 477-489.
9. Parry, D. A. (1951). Factors determining the temperature of terrestrial arthropods in sunlight. *Journal of Experimental Biology*, *28*(4), 445-462.
10. Cerdá, X. (2001). Behavioural and physiological traits to thermal stress tolerance in two Spanish desert ants.
11. McMeeking, R. M., Arzt, E., & Wehner, R. (2012). Cataglyphis desert ants improve their mobility by raising the gaster. *Journal of theoretical biology*, *297*, 17-25.
12. Withrow RB, Withrow AP. Generation, control and measurement of visible and near-visible radiant energy. Radiat Biol. 1956; 3: 125–258.
13. Kettlewell, B. (1973). The Evolution of Melanism, the Study of a Recurring Necessity with Special Reference to Industrial Melanism in the Lepidoptera. Clarendon
14. Trullas, S. C., van Wyk, J. H., & Spotila, J. R. (2007). Thermal melanism in ectotherms. *Journal of Thermal Biology*, *32*(5), 235-24
15. Clusella-Trullas, S., Terblanche, J. S., Blackburn, T. M., & Chown, S. L. (2008). Testing the thermal melanism hypothesis: a macrophysiological approach. *Functional ecology*, *22*(2), 232-238.
16. Zeuss, D., Brandl, R., Brändle, M., Rahbek, C., & Brunzel, S. (2014). Global warming favours light-coloured insects in Europe. *Nature Communications*, *5*, 3874.
17. Available from http://www.antweb.org. Accessed 3 October 2018
18. Peeters, C., Molet, M., Lin, C. C., & Billen, J. (2017). Evolution of cheaper workers in ants: a comparative study of exoskeleton thickness. *Biological Journal of the Linnean Society*, *121*(3), 556-563.
19. Shi, N. N., Tsai, C. C., Camino, F., Bernard, G. D., Yu, N., & Wehner, R. (2015). Keeping cool: enhanced optical reflection and heat dissipation in silver ants. *Science*, aab3564.
20. Edney, E. B. (1971). The body temperature of tenebrionid beetles in the Namib Desert of southern Africa. *Journal of experimental Biology*, *55*(1), 253-272.
21. Turner, J. S., & Lombard, A. T. (1990). Body color and body temperature in white and black Namib. *Journal of Arid Environments*, *19*, 303-315.
22. Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual review of physiology*, *61*(1), 243-282.
23. Garbuz, D., Evgenev, M. B., Feder, M. E., & Zatsepina, O. G. (2003). Evolution of thermotolerance and the heat-shock response: evidence from inter/intraspecific comparison and interspecific hybridization in the virilis species group of Drosophila. I. Thermal phenotype. *Journal of Experimental Biology*, *206*(14), 2399-2408.
24. Tomanek, L., & Somero, G. N. (1999). Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus Tegula) from different thermal habitats: implications for limits of thermotolerance and biogeography. *Journal of Experimental Biology*, *202*(21), 2925-2936.
25. Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & development*, *12*(24), 3788-3796.

26. Zatsepina, O. G., Ulmasov, K. A., Beresten, S. F., Molodtsov, V. B., Rybtsov, S. A., & Evgen'Ev, M. B. (2000). Thermotolerant desert lizards characteristically differ in terms of heat-shock system regulation. *Journal of Experimental Biology*, *203*(6), 1017-1025.
27. Lund, S. G., Ruberté, M. R., & Hofmann, G. E. (2006). Turning up the heat: the effects of thermal acclimation on the kinetics of hsp70 gene expression in the eurythermal goby,
28. Nguyen, A. D., Gotelli, N. J., & Cahan, S. H. (2016). The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC evolutionary biology*, *16*(1), 15.
29. Amin, J. A. H. A. N. S. H. A. H., Ananthan, J. A. Y. A. K. U. M. A. R., & Voellmy, R. I. C. H. A. R. D. (1988). Key features of heat shock regulatory elements. *Molecular and cellular biology*, *8*(9), 3761-3769.
30. de Jong, W. W., Caspers, G. J., & Leunissen, J. A. (1998). Genealogy of the α-crystallin—small heat-shock protein superfamily. *International journal of biological macromolecules*, *22*(3-4), 151-162.
31. Sondermann, H., Scheufler, C., Schneider, C., Höhfeld, J., Hartl, F. U., & Moarefi, I. (2001). Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. *Science*, *291*(5508), 1553-1557.
32. Bettencourt, B. R., & Feder, M. E. (2002). Rapid concerted evolution via gene conversion at the Drosophila hsp70 genes. *Journal of molecular evolution*, *54*(5), 569-586.
33. Sørensen, J. G., Kristensen, T. N., & Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, *6*(11), 1025-1037.
34. Chen, B., Zhong, D., & Monteiro, A. (2006). Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. *BMC genomics*, *7*(1), 156
35. Daugaard, M., Rohde, M., & Jäättelä, M. (2007). The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS letters*, *581*(19), 3702-3710.
36. Shopland, L. S., Hirayoshi, K., Fernandes, M., & Lis, J. T. (1995). HSF access to heat shock elements in vivo depends critically on promoter architecture defined by GAGA factor, TFIID, and RNA polymerase II binding sites. *Genes & development*, *9*(22), 2756-2769.
37. Fuda, N. J., Guertin, M. J., Sharma, S., Danko, C. G., Martins, A. L., Siepel, A., & Lis, J. T. (2015). GAGA factor maintains nucleosome-free regions and has a role in RNA polymerase II recruitment to promoters. *PLoS genetics*, *11*(3), e1005108.
38. Miller, S. B., Ho, C. T., Winkler, J., Khokhrina, M., Neuner, A., Mohamed, M. Y., ... & Mogk, A. (2015). Compartment-specific aggregases direct distinct nuclear and cytoplasmic aggregate deposition. *The EMBO journal*, *34*(6), 778-797.
39. Lüders, J., Demand, J., & Höhfeld, J. (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *Journal of Biological Chemistry*, *275*(7), 4613-4617.
40. Bilyk, K. T., Vargas-Chacoff, L., & Cheng, C. H. C. (2018). Evolution in chronic cold: varied loss of cellular response to heat in Antarctic notothenioid fish. *BMC evolutionary biology*, *18*(1), 143.
41. Evgen'Ev, M. B., Garbuz, D. G., & Zatsepina, O. G. (2014). *Heat shock proteins and whole body adaptation to extreme environments*. Springer.
42. Rinehart, J. P., Hayward, S. A., Elnitsky, M. A., Sandro, L. H., Lee, R. E., & Denlinger, D. L. (2006). Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proceedings of the National Academy of Sciences*, *103*(38), 14223-14227.
43. Wehner, R., Marsh, A. C., & Wehner, S. (1992). Desert ants on a thermal tightrope. *Nature*, *357*(6379), 586.